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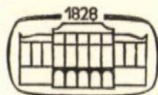
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THE INDIA-INK IMMUNO-REACTION AS A NEW METHOD FOR THE RAPID DIAGNOSIS OF YEAST STRAINS

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Summary. The authors' earlier immunofluorescence method has been improved for the adequate serological identification of yeasts by the application of the India-ink immuno-reaction. The method has been adapted for the rapid identification of the most frequent pathogenic yeast, *Procanidida albicans*.

Application of serological methods is necessary in medical mycology and in the other branches of mycology. Adequate serological procedures could avoid the laborious and time-consuming methods used for the identification of yeasts and yeast-like fungi (subsequently, yeasts) [5]. The serological relatedness of yeast species, however, represents a great difficulty for the technical realization of these methods (complicated procedures, monospecific sera, etc.). Therefore, in medical mycology other rapid diagnostic procedures are often applied for the identification of frequent species (for example, *Procanidida albicans*). Such methods are TTC and bismuth agar, different chlamydo-spore agar media [7], filament production tests in human sera or egg white [8], etc. These procedures are used also as screening tests.

However, all these methods, such as the TTC and bismuth agar assay, need an incubation period of 24-48 hours. Those requiring 1-2 hours enable the identification of a single species only, i.e. of *Pc. albicans* (synonym *Candida albicans* [6]). The same is the case with the serum and the egg white reactions. Moreover, their application as a screening test is limited by their expensiveness.

We have published a version of the immunofluorescence method [3] for the identification of yeasts which ensured the diagnosis in about 30 minutes and specificity was also achieved by simple means, as instead of monospecific sera, serum dilutions, yielding the same selective reaction, were applied. However, the use of this method was also limited because of the need of a fluorescence microscope and the expensive, hardly storable conjugate. The India-ink immuno-reaction has been developed for the elimination of the drawbacks of the immunofluorescence method [1, 2]. Experience concerning the mycological adaptability of the method and its usefulness as a means for the rapid diagnosis of *Pc. albicans*, the most frequent pathogenic yeast species in Hungary [10], are discussed in the following.

Materials and methods

The procedures used for the production of rabbit immune sera, cultivation of the strains, preparation of smears were the same as in our earlier study [3]. The India-ink immuno-reaction has been carried out according to GECK [1, 2] as follows.

(a) Smears of 48-hour cultures of adequate yeast strains were dried and fixed with heat.

(b) One drop of liquid India-ink ("Holló" Chinese India-ink, Politur és Vegyi Termékek Gyára, Budapest) was mounted on the smears with one drop of an adequate dilution of an anti-*Pc. albicans* rabbit immune serum, then mixed with a wire-loop and incubated in a moist chamber at room temperature for 5 minutes.

(c) Following the incubation period the smears were washed in isotonic NaCl solution mixed with tap water (400 + 600 ml), under medium pressure by means of a ball pipette.

Direct evaluation of the method and the photomicrographs were performed with a Zeiss NfpK microscope at about 600 × magnification. The basis of the evaluation was the following arbitrary scale (see also Table I and Fig. 1):

- only the light greenish-grey contour of the cells is visible;
- ± the cells are hardly visible, aggregates of India-ink as scattered black spots on the cell wall can be observed;
- + an interrupted black India-ink deposit covers the wall of the cells;
- ++ a thin continuous black coat covers the wall of the cells, within the contour of the India-ink deposit the cell is greyish-white in colour;
- +++ the wall of the cells has an intensive black contour because of the India-ink deposit, the cell itself is greyish-brown in colour;
- ++++ the wall of the cells appears as a broad intensive black contour, the cell is deep brown in colour.

Results and discussion

On the basis of literary data [9] and our own study [3] ten species have been selected for the evaluation of the method. These species were partly related serologically to *Pc. albicans*, partly unrelated ones. The results are summarized in Table I.

Table I

India-ink immuno-reaction carried out with different dilutions of an anti-*Pc. albicans* 360/1960 rabbit immune serum and different yeast strains

Species and designation of strains	Serum dilution						
	1:5	1:10	1:20	1:40	1:80	1:160	1:320
<i>Pc. albicans</i> 85/1957	++++	++++	++++	++++	++++	++	+
<i>Pc. tropicalis</i> 302/1964	++++	++++	++++	+++	++	+	—
<i>Pc. stellatoidea</i> 29-64	++++	++++	++++	++	+	+	—
<i>C. beverwijkii</i> 478/1961	++++	++++	+++	++	±	±	—
<i>S. cerevisiae</i> CXCII/1967	++++	++++	++	+	±	±	—
<i>C. reukaufii</i> 405	+++	++	++	+	±	±	—
<i>N. slovaca</i> 771	+	±	—	—	—	—	—
<i>C. utilis</i> 287/1964	±	—	—	—	—	—	—

Pc. = *Procandida*; *C.* = *Candida*; *S.* = *Saccharomyces*; *N.* = *Nadsonia*, the synonym of *C. humicola* (Lodder 1970)

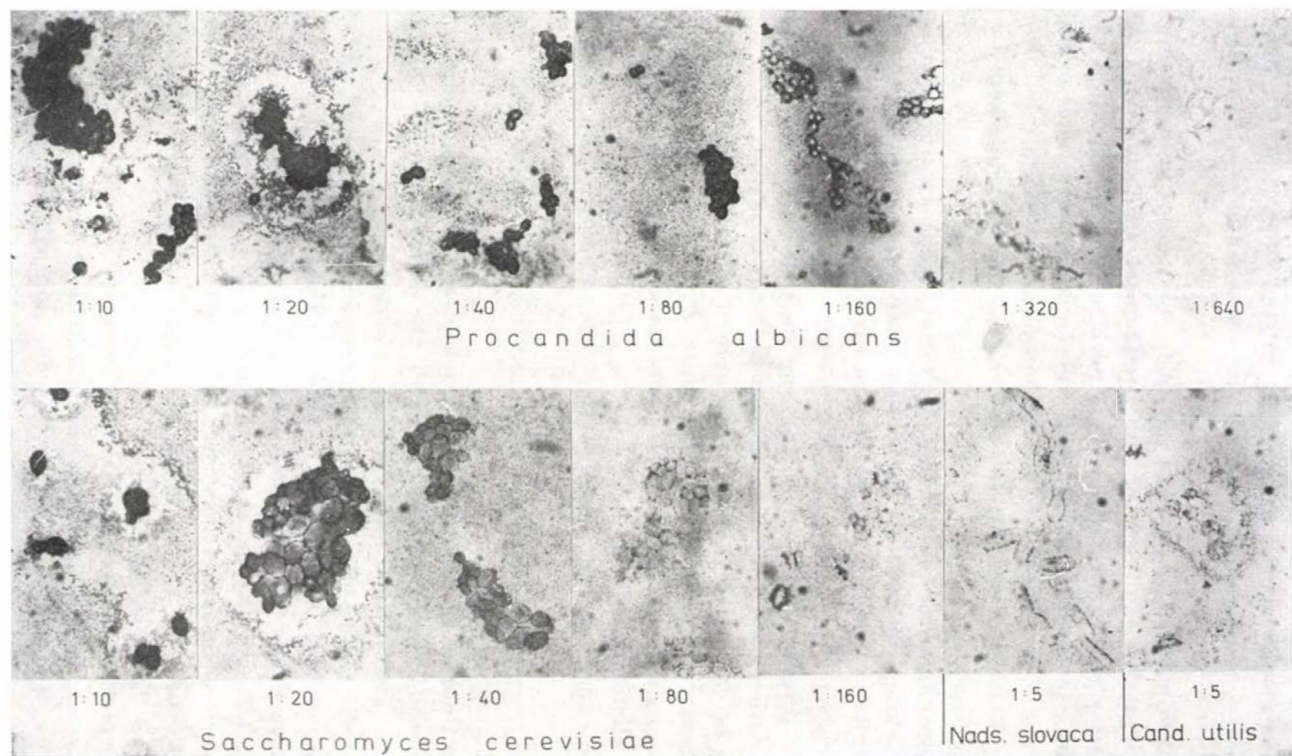


Fig. 1. India-ink immuno-reactions of yeasts with different dilutions of anti-*Pc. albicans* rabbit immune serum. (The strains and the serum are the same as in Table I, the numbers below the Figures represent the serum dilutions. The magnification is 400, except for the two Figures in the right upper corner, where the magnification is $\times 770$)

Our results show that it is easy to differentiate *Pc. albicans* from other species, since the dilution of the immune sera decreases the positivity of the cross-reactions displayed by antigenically related species [3, 9]. The critical serum dilution — allowing a differentiation — was in the present examinations 1 : 80.

Further dilution of the immune sera beyond this optimum level (ensuring selectivity) is useless, in view of the spontaneous (non-immune) India-ink binding capacity of certain species. This problem will be discussed elsewhere in detail. Our earlier work dealing with the immunofluorescence examination of 102 yeast species revealed that *Pc. albicans* can be differentiated from other fungi by the dilution of an anti-*Pc. albicans* rabbit immune serum by means of a microagglutination method [3]. Dilution of the immune sera was found to increase the specificity of the reaction. Relevant results are presented in Fig. 1.

The working-dilution of the serum used in the India-ink immunoreaction is 1 : 80 — which is in good agreement with the results yielded by the immunofluorescence method for the same serum [3]. This means that the use of this single dilution may select the smears containing *Pc. albicans*. The microagglutination titre of the stored sera does not change.

It may be concluded that our new method offers the same results as the immunofluorescence method, but this method is much more simple, more rapid and less expensive. The following drawbacks of the immunofluorescence method are eliminated by it.

(a) The expensive fluorescence microscope and the dark room are not needed.

(b) The expensive fluorescent dyes and the preparation of the conjugate are avoided.

(c) The fluorescence method is a much more time-consuming procedure (the direct method requires 20–30 minutes, the indirect one 40–60 minutes). The conjugate can be stored only for a short period.

(d) The disturbing effect of autofluorescence is eliminated and there is no need of a versatile investigator to evaluate the results.

Our method has the following practical advantages:

(a) *Pc. albicans* is the most frequent pathogenic yeast in systemic mycoses as well as in dermatomycoses [4]. In Hungary, the pathogens of systemic mycotic diseases are yeasts in 66%, and among them *Pc. albicans* is the pathogenic agent in 50% [10].

(b) The method is rapid and simple, therefore *Pc. albicans* can be identified in cultures as well as in exudates even in the case of a great number of screening tests. Execution of the method does not call for special knowledge.

(c) The method is not expensive, since a single rabbit may yield about 50 ml of immune serum. If we are working with a dilution of 1 : 100 — if necessary, sera with higher titres can easily be prepared — and with 0.1 ml

volume of the diluted serum for every smear, then 50 000 examinations can be accomplished with the serum of a single rabbit (stored sera do not lose their activity).

(d) The dilution ensures the specificity in itself without any procedure of absorption. Therefore, the use of adequate absorbing strains and the absorption procedure (which causes a serum loss) can be avoided.

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ELECTRON MICROSCOPIC LOCALIZATION OF ALKALINE PHOSPHATASE IN AN ADENINE-DEPENDENT MUTANT OF BACILLUS ANTHRACIS

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(Received March 2, 1972)

Summary. The cytochemical localization of alkaline phosphatase and the structure of the cell wall were studied by electron microscopy in a prototrophic strain of *Bacillus anthracis* Vollum R and in its adenine-dependent auxotroph showing derepressed alkaline phosphatase synthesis. Enzyme localization could be observed in the area between the cell wall and the cytoplasmic membrane in both strains. In the adenine-dependent mutant, however, a more pronounced enzyme activity with a loosening of the layers of the cell wall could be observed. The data obtained may in part explain the decreased capacity of the mutant to propagate in the animal organism.

Several mutants with varying degrees of auxotrophism were isolated from the Vollum R (non-capsulogenic mutant) strain of *Bacillus anthracis* by Ivánovics *et al.* [1]. Similar to the prototroph strain, the capsulated variants, except the adenine auxotrophs, of the isolated strains exhibited pathogenicity in the mouse [2, 3]. Alkaline phosphatase activity was higher in the adenine-dependent mutants than in the prototrophic or in the pyrimidine and vitamin-dependent strains [4]. The repressibility of the enzyme was the same in the prototrophic revertant of the adenine-dependent strain and in the parent strain.

It seemed reasonable to suppose that, due to its derepressed alkaline phosphatase activity, the mutant was able to synthesize none or only a small amount of the phosphorous components essential for the formation of the bacterial cell wall. Our attempts to detect such a phosphorous polymer, *viz.* teichoic acid, have failed since this compound is not present in the cell wall either of the adenine-auxotrophic or of the prototrophic strains [5].

SMIRNOVA *et al.* [6] have shown that the formation of the cell wall in *B. subtilis* strains with high phosphate activity is disturbed. The layers of the disturbed cell wall become loosened and some of them separated. The presence of alkaline phosphatase could be demonstrated cytochemically by electron microscopy in the loosened cell wall. DONE *et al.* [7] found the enzyme in the area between the cytoplasmic membrane and the cell wall in *E. coli* and WOOD and TRISTRAM [8] showed it in *B. subtilis*. Studying the effect of derepressed alkaline phosphatase on morphological changes in *E. coli*, KUSHNAREV observed a pronounced desquamation of the lipoprotein layer of the cell wall [9]. GLEW and HEATH [10] found that, as a result of the derepressed alkaline

phosphatase activity, a high portion of the enzyme was excreted and no accumulation between the cytoplasmic membrane and the cell wall occurred in *Streptococcus sodonensis*.

The above data have prompted us to study in the above mentioned two strains of *B. anthracis* the electron microscopic localization of alkaline phosphatase and other morphological changes possibly connected with the de-repressed enzyme synthesis.

Materials and methods

Bacterial strains. The non-capsulogenic (VC⁻) mutant Vollum of *B. anthracis* and its 23C⁻ ade⁻H-3 adenine auxotrophy described by IVÁNOVICS *et al.* [1] were used.

Medium. Lactalbumin hydrolysate as described in [4] was used. For the elimination of phosphorus from the lactalbumin hydrolysate solution, barium hydroxide was added and then the excess barium was removed by sulphuric acid. The solution of the lactalbumin hydrolysate was filtered through a G-5 glass filter after both barium hydroxide and sulphuric acid treatment.

Cultivation. After adequate completion [4], the medium was supplemented with K₂HPO₄ to ensure a phosphate concentration of 1.0 mM. At this phosphate concentration the synthesis of alkaline phosphatase is already repressed in the wild strain, but is still de-repressed in the adenine-dependent mutant. To cultivate the adenine-dependent mutant, 10 µg/ml adenine was also added to the medium. Ten ml aliquots of the medium were distributed into 100 ml Erlenmeyer flasks each of which was then inoculated with 10⁵ spores. The flasks were incubated with airing in a shaker in a water bath at 37°C for 16 hours. After this incubation period, the optical density of the bacterial cultures was determined by a densitometer (Magnephot II, Orion, Budapest, Hungary).

Quantitative determination of alkaline phosphatase. Ten ml bacterial culture was centrifuged in cold at 3500 r.p.m. for 15 minutes. The pellet was washed once with physiological saline and then it was resuspended in 0.1 M sodium cacodylate buffer (pH 7.4) and diluted to reach an optical density of 0.6. The suspension contained 0.300 mg/ml dry bacteria. To 2 ml of the bacterial suspension, 2 ml substrate solution was added. The composition of the substrate solution was the same as that conventionally used for electron microscopic studies [6] except that instead of 2% β-glycerophosphate 2% p-nitrophenyl phosphate was used for supplementation. The samples were incubated in a water bath at 37°C for 20 minutes. The enzyme reaction was stopped by adding 1.0 ml of 1.0 M NaOH to the tubes which were then centrifuged in cold at 3500 r.p.m. for 20 minutes. The optical densities of the supernatants were determined with a Beckman DU spectrophotometer at 410 mµ. Enzyme activity was expressed in terms of the amount of p-nitrophenol formed in 20 minutes: 1 enzyme unit = 1.0 mµ M paranitrophenol released by 1 ml bacterial suspension in 20 minutes.

Electron microscopic localization of alkaline phosphatase. The bacterial culture was centrifuged in cold at 3500 r.p.m. for 20 minutes and the pellet was resuspended in 2.0 ml of 0.1 M cacodylate buffer (pH 7.4). After the addition of 2.0 ml of 12.5% glutaraldehyde (25% glutaraldehyde commercially available was diluted to 1 : 2 with 0.1 M cacodylate buffer pH 7.4), the suspension was pre-fixed in ice under vigorous shaking. After centrifugation, the pellet was washed twice in cacodylate buffer and three times in 0.1 M tris-HCl buffer pH 8.5.

After glutaraldehyde treatment, incubation with the substrate and cobalt treatment were performed as described by DONE *et al.* [6]. Subsequently, the bacteria were centrifuged and the pellet was washed three times in 0.1 M cacodylate buffer (pH 7.4). Post-fixation for 3 hours was made in cold with 1.0% osmium tetroxide in cacodylate buffer. In contrast to the method used earlier [11], when fixation with glutaraldehyde and osmium tetroxide had been made at pH 6.0, fixation in the present experiments was performed at pH 7.2–7.4. The reason for changing the pH was that alkaline phosphatase may become partially inactivated at an acid pH and part of the already precipitated cobalt phosphate may become redissolved. Osmium tetroxide post-fixation was followed by dehydration through an alcoholic series. Staining was carried out during dehydration, keeping the samples in a solution of saturated uranyl acetate in 70% alcohol at 4°C overnight. The samples were embedded in Durcupan [12]. The blocks were sectioned by an LKB ultramicrotome. The sections were mounted on a grid covered with Formvar film and studied with a JEM-100B electron-microscope.

For cobalt determination, the ammonium thiocyanate method [12] was used.

Results

First, changes induced by glutaraldehyde pre-fixation in the alkaline phosphatase activity of the cells were examined. It was found that the enzyme activity decreased to 20% of the original level in *B. anthracis* VC- cells and to 19% of the original level in the adenine-dependent mutant in 5 minutes after treatment with 6.5% glutaraldehyde; no further decrease occurred in the next

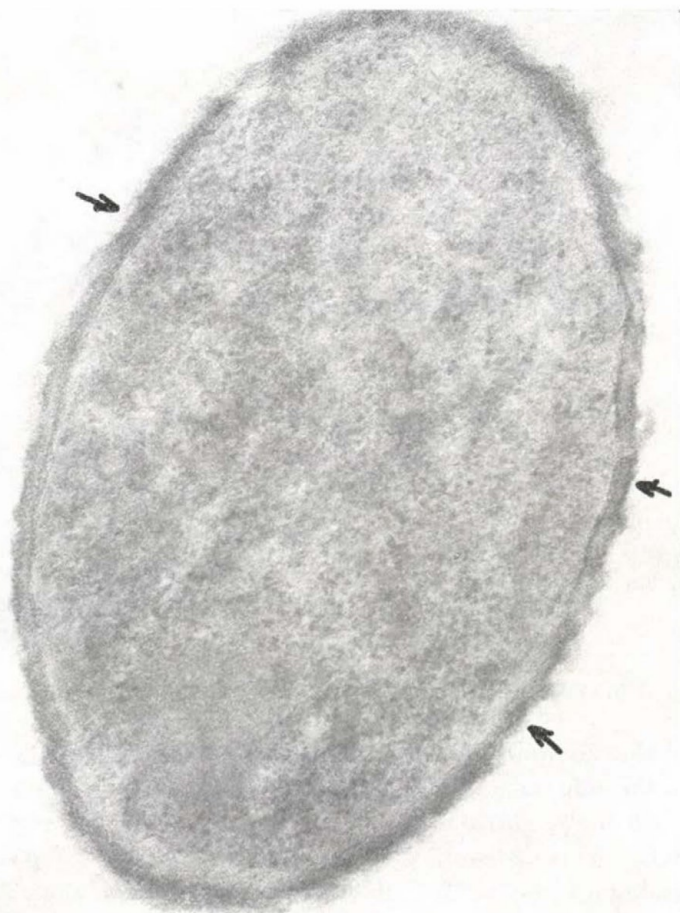


Fig. 1. Section of *B. anthracis* 23C⁻ ade⁻II-3 stained with cobalt nitrate to detect alkaline phosphatase ($\times 75\,000$)

90 minutes. This effect was then studied at two different glutaraldehyde concentrations, 2.5 and 6.5%. Both concentrations induced the same rate of decrease in enzyme activity in both strains.

Next, the effect of different periods of pre-fixation with 6.5% glutar-

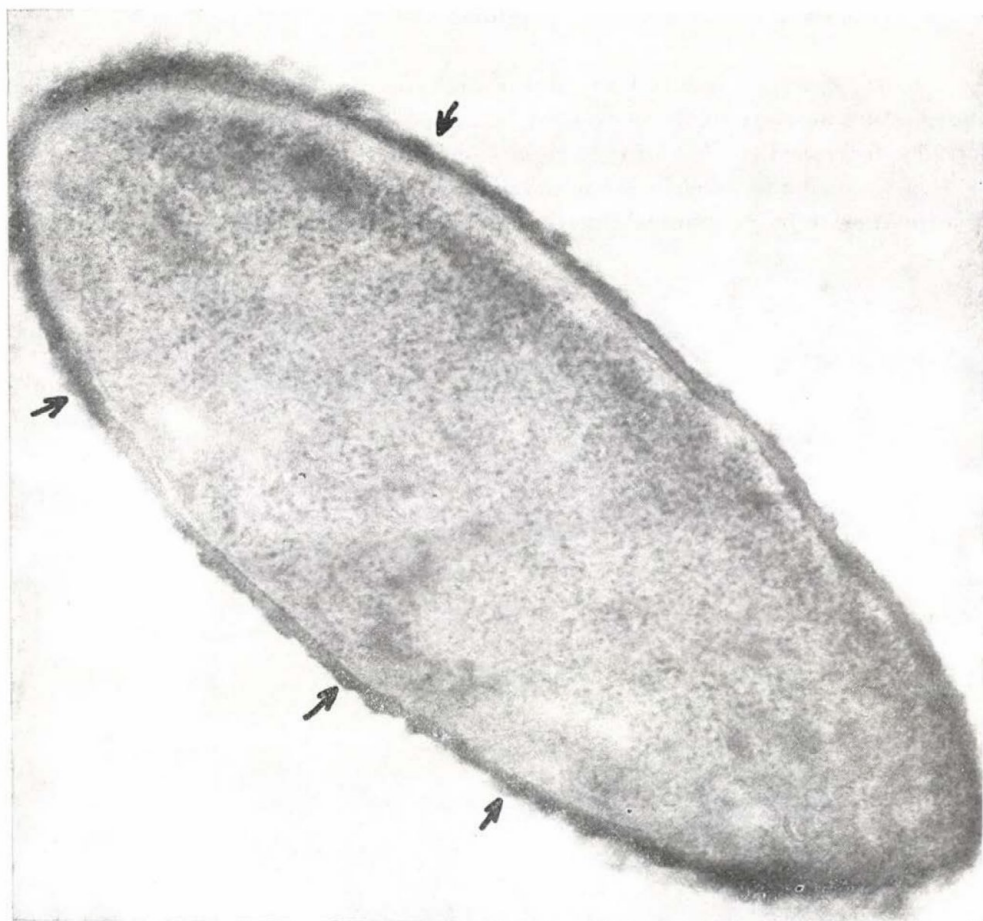


Fig. 2. Section of *B. anthracis* 23C⁻ ade⁻ II-3 stained with uranyl acetate and cobalt nitrate to detect alkaline phosphatase ($\times 62\,500$)

aldehyde on the amount of cobalt phosphate precipitating in bacteria incubated with the substrate was examined. Aliquots of the bacterial suspension were fixed with 6.5% glutaraldehyde for periods of 30, 60 and 90 minutes, incubated with the substrate, then treated with a 2% solution of cobalt nitrate according to the method described above. After centrifugation and washing, the pellet was suspended in 3 ml of 1.0 M HCl and the suspension was let to stand for 19 hours and shaken occasionally. After repeated centrifugation, 1 ml of each supernatant was used for cobalt determination [13]. The results showed that in bacteria pre-fixed for 30 minutes, 88 μg , while in those pre-fixed for 60 and 90 minutes, 41 and 42 μg cobalt, respectively, could be detected. Since maximum precipitation was obtained after 30 minutes and both the 60 and the 90 minutes pre-fixation reduced this maximum to 47%, 30 minutes prefixation was used in each case.

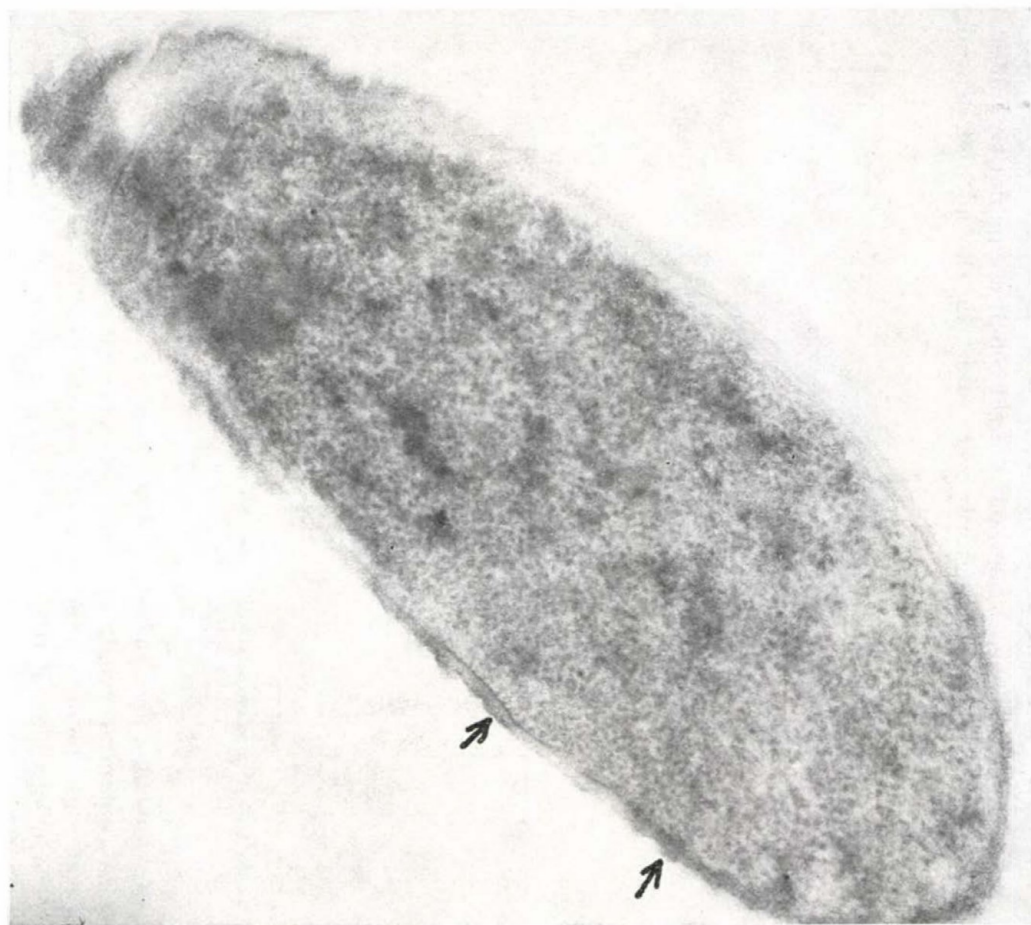


Fig. 3. Section of *B. anthracis* VC⁻ stained with uranyl acetate and cobalt nitrate to detect alkaline phosphatase ($\times 62\,500$)

Samples only post-fixed with osmium tetroxide or samples post-fixed with osmium tetroxide and stained with uranyl acetate were used. For electron microscopic studies, with the former method cobalt precipitation yielded a slight contrast (Fig. 1), while in samples stained with uranyl acetate the sites of cobalt precipitation showed a more intensive contrast (Fig. 2).

Next, the localization of alkaline phosphatase in the prototrophic strain of *B. anthracis* was studied (Fig. 3). Consistent with the low enzyme activity determined by the enzymatic method, a slight cobalt accumulation and intact cell wall and membrane structures were seen. In the adenine-dependent

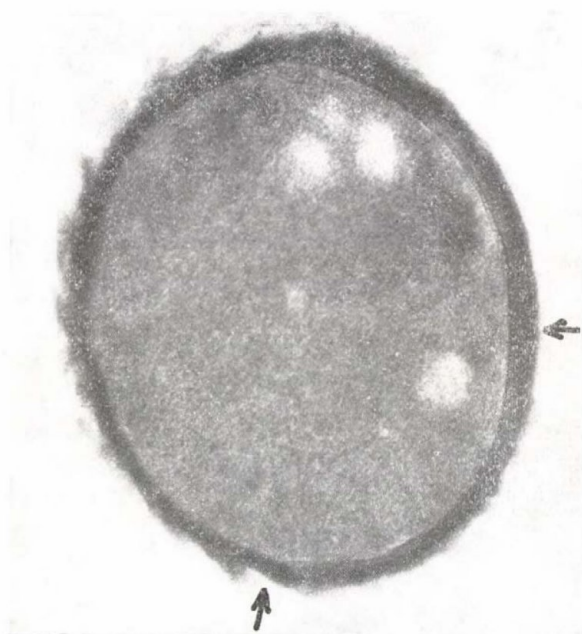


Fig. 4. Section of *B. anthracis* 23C⁻ ade⁻II-3 stained with uranyl acetate and cobalt nitrate to detect alkaline phosphatase ($\times 50\,000$)

mutant showing a high enzyme activity as a result of the derepressed enzyme synthesis, intensive cobalt accumulation between the cell wall and the cell membrane was found in large areas (Figs 4 and 5). In the adenine-auxotrophic bacteria, a loosening of the cell wall was also observed in several instances (Fig. 6). This morphological change may be explained by the derepressed alkaline phosphatase synthesis in these cells [5].

In order to increase the contrast, the electron micrograms presented have been taken with the electron microscope slightly overfocussed. Accumulation of the enzyme could be observed between the cytoplasmic membrane and the cell wall (Fig. 7) where a structureless electron dense material was seen between

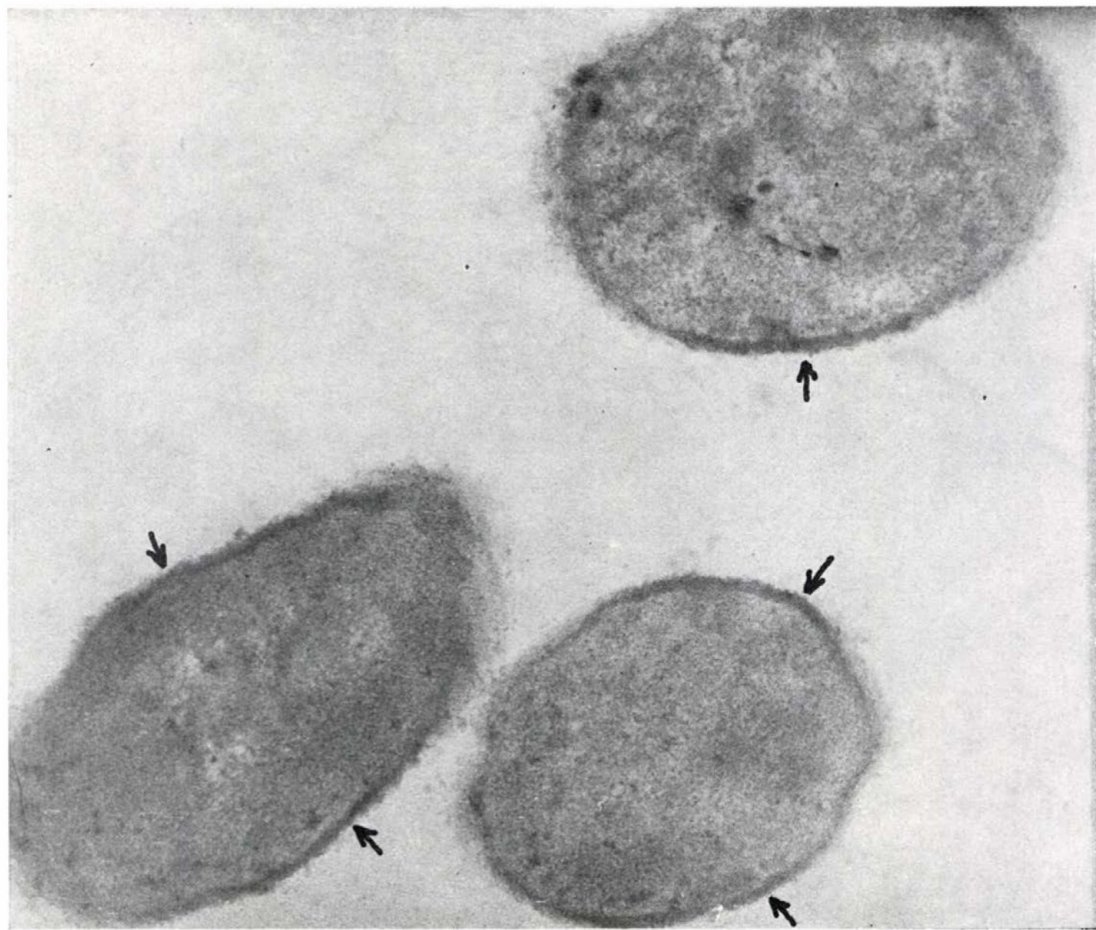


Fig. 5. Section of *B. anthracis* 23C⁻ ade⁻II-3 stained with uranyl acetate and cobalt nitrate to detect alkaline phosphatase ($\times 50\ 000$)



Fig. 6. Section of *B. anthracis* 23C⁻ ade⁻II-3 stained with uranyl acetate and cobalt nitrate to detect alkaline phosphatase ($\times 100\ 000$)

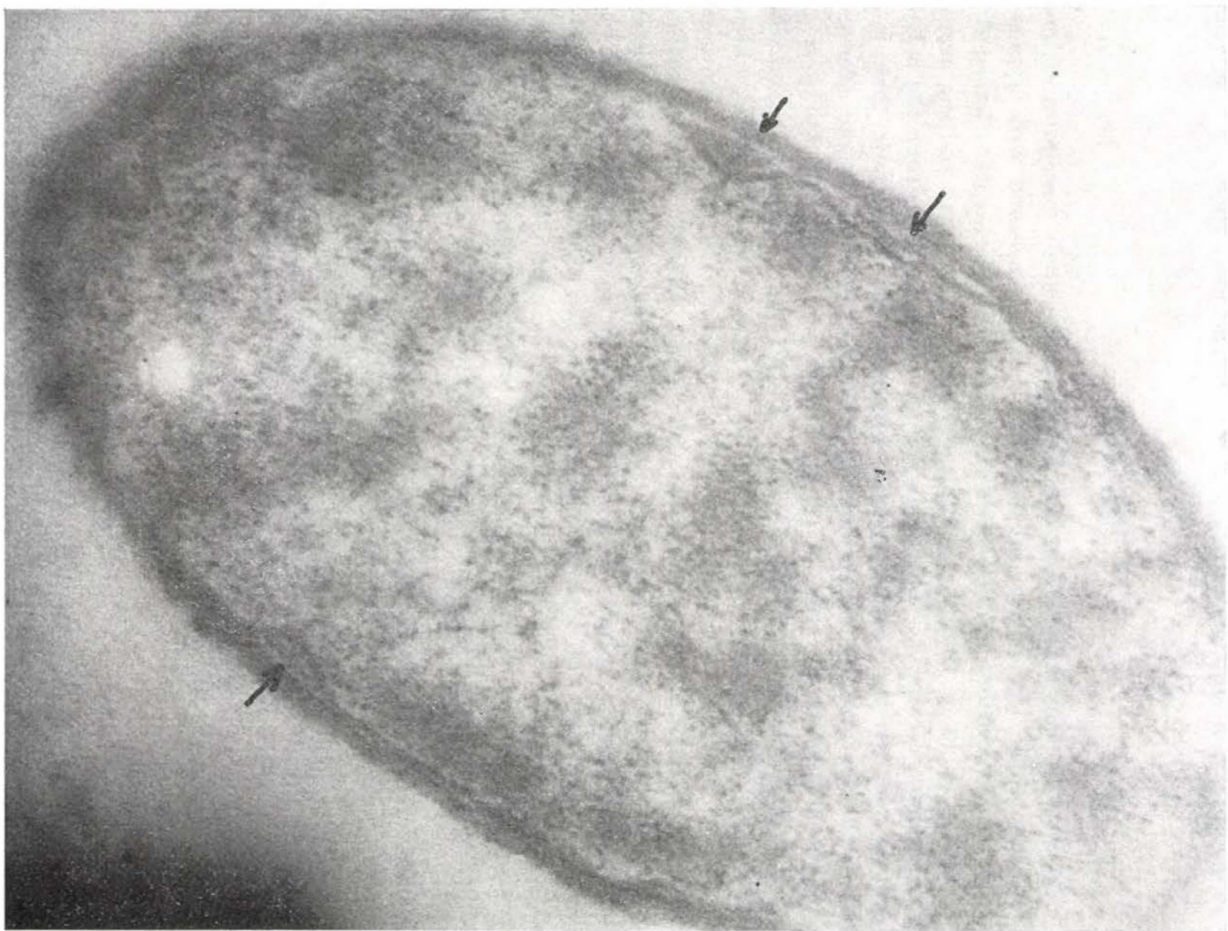


Fig. 7. Section of *B. anthracis* 23C⁻ ade⁻II-3 stained with cobalt nitrate to detect alkaline phosphatase ($\times 100\ 000$)

the infolded cytoplasmic membrane and the cell wall. Therefore some samples were not stained with uranyl acetate in order to prevent the precipitation of the strongly electron dense cobalt-uranyl complex and to improve the visibility of the membrane and of the cell wall and the area between them.

Discussion

It may be concluded from the above that cobalt precipitation corresponding to the site of enzyme activity can be observed with a better contrast in preparations stained with uranyl acetate. The explanation of this phenomenon might be that the precipitated cobalt phosphate and the uranyl salt are forming a complex [14] which is more electron dense than the cobalt phosphate itself. Of the enzyme activity present in living bacteria, 20% persisted in the prototrophic strain and 19% could be detected in the adenine-dependent mutant as a result of the applied fixation method. DONE *et al.* found a residual enzyme activity of 31% in *E. coli* under similar experimental conditions [7]. As compared to the prototrophic strain, the adenine-dependent mutant of *B. anthracis* exhibiting derepressed alkaline phosphatase synthesis underwent different morphological changes. These changes were consistent with those found by other authors [6–9]. Our electron-microscopic observations suggesting an injury of the bacterial cell, may partly explain the finding of IVÁNOVICS *et al.* that the capsulated variant of the mutant, like the prototroph, is capable of toxin production and is incapable of propagation even after the addition of exogenous adenine when injected in mice, and that the number of injected bacteria decreases to half in 8 hours after the injection [2, 3].

The derepressed state of alkaline phosphatase synthesis in the adenine-dependent mutant possibly induces alterations in a number of biochemical processes which in turn result in a significant decrease in the rate of bacterial propagation. The increased rate of synthesis and the wide substrate range of the enzyme lead to defects of bacterial metabolism by the decomposition of different organic phosphate esters. The intensive accumulation of the enzyme and the mechanism of its excretion may then induce changes in the cell structure.

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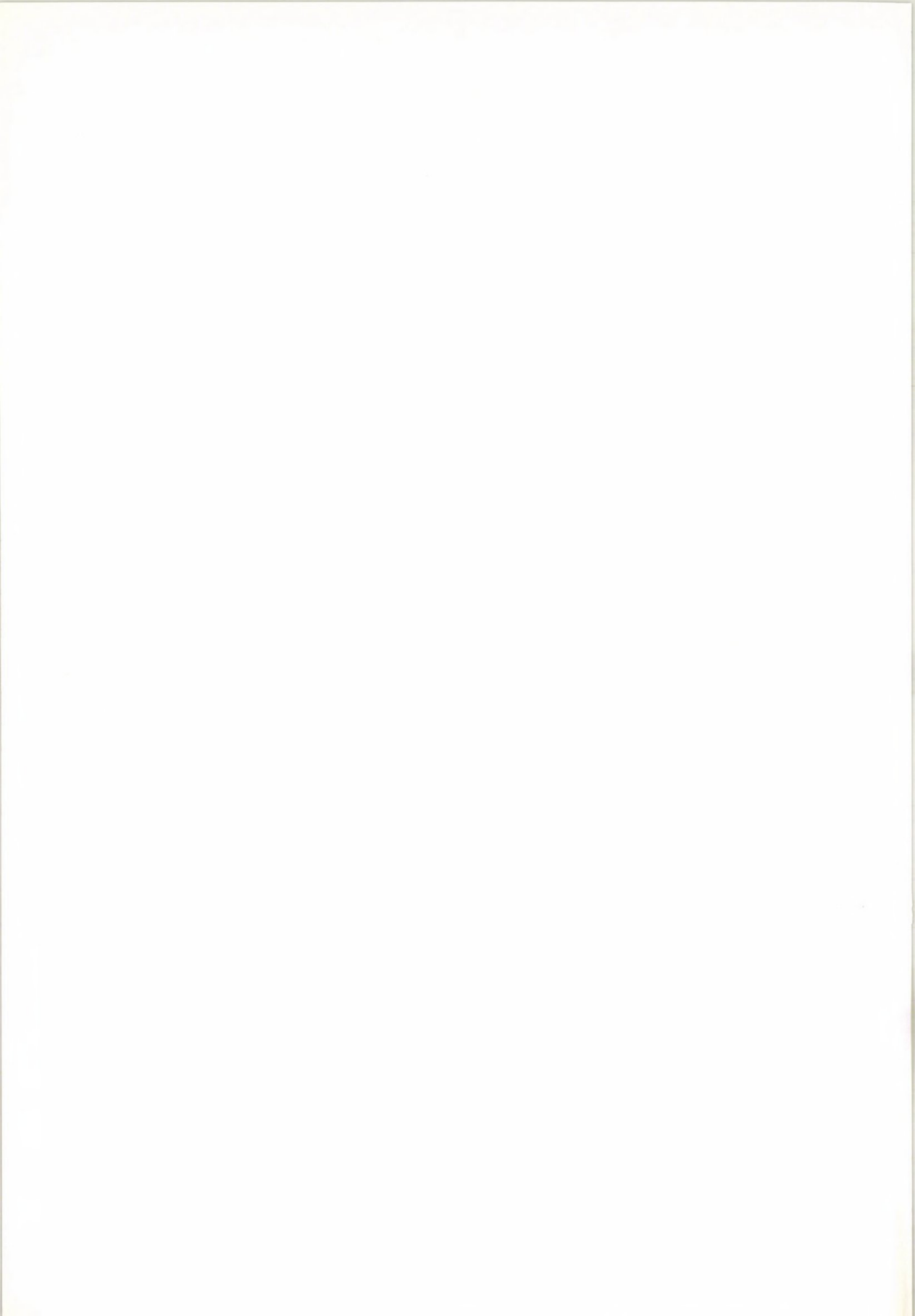
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SPECIFIC ORAL PREVENTION OF INFANTILE ENTERITIS

III. EXPERIMENTS WITH CORPUSCULAR VACCINE*

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(Received March 28, 1972)

Summary. (i) The immunizing capacity of Boivin antigen and formalinized corpuscular vaccine prepared from *Escherichia coli* O111 has been compared in mouse experiments.

(ii) The degree of active immunity was associated with the germ equivalency of corpuscular vaccine and Boivin antigen. Passive protection tests with sera of actively immunized mice have confirmed this finding. The favourable effect of revaccination with small amounts of vaccine and the production of copro-antibodies were also demonstrated.

(iii) In view of the good results of mouse experiments a comparative examination of polyvalent *E. coli* (O111, O55 and O86) Boivin and corpuscular vaccine was performed in infants between 6 weeks and 6 months of age.

(iv) Infants vaccinated with corpuscular vaccine (9×10^{11} cells of each component) and with equivalents of Boivin antigens (9 mg of each component) showed the same increase in protective serum antibodies. After revaccination with small amounts of both kinds of vaccine, the titres rose higher. The protective effect of serum from infants vaccinated on each of 6 consecutive days was twice higher than of serum from infants vaccinated 3 times at 5-day intervals.

(v) Infants receiving a total dose of 1.2×10^{12} cells of each component and the equivalent Boivin antigen (12 mg of each component) during 6 days showed a still higher rise in protective antibody titre which could not be increased with revaccination.

(vi) Infants vaccinated on 6 consecutive days developed antibodies identical in level to those in infants receiving the same total dose within 3 days.

(vii) Although the 3-dose vaccination (3.6×10^{12} cells or 36 mg Boivin antigen *in toto*) loaded the infants with a large amount of endotoxin, no toxic symptoms were observed.

(viii) In view of the laboratory results and of experience obtained in a hospital outbreak, an adequately controlled wide-scale trial of the vaccine seems justified.

It has been shown by us that small oral doses of Boivin antigen prepared from *Escherichia coli* strains associated with infantile enteritis are able to protect mice against intraperitoneal infection with the homologous culture [1]. In these experiments general immunity was demonstrated by high levels of protective antibodies in the sera of mice and the development of local immunity was indicated by the presence of copro-antibodies in intestinal extracts. The level of protective antibodies decreased rapidly, but minimal booster doses maintained the immunity practically as long as required. Vaccination of 62, mainly dystrophic, infants resulted in the production of both serum and copro-antibodies. The titre induced by a small oral dose could further be increased by administering repeated weekly doses, but the maximum response induced by an optimum dose could not be raised by further vaccinations. In

* We offer this paper for the 60th birthday of Prof. Otto Westphal.

the latter case the booster doses served only for maintaining the maximum titre [2, 3].

For economic reasons the question arose as to whether an adequate immunity is induced by killed corpuscular vaccine. The present paper gives an account of parallel immunizations with bacterial suspension and with Boivin extract of mice and of infants.

Materials and methods

Bacterial strains were the same as described in previous papers [1—3].

Preparation of Boivin extract and of tabletted vaccine was carried out as previously [2, 3].

Bacterial suspensions were prepared by fermentation in casein hydrolysate medium from *E. coli* O111 : K58(B4), O55 : K59(B5) and O86 : K61(B7). After the addition of 0.3% formalin, the suspensions were kept at 5°C overnight, then washed and tested for sterility. Each suspension was adjusted to the required density by the use of the NIH/Human opacity standard, then equal portions were mixed and ampouled aseptically.

Oral immunization of mice was performed with a metal cannula fitted to a syringe. Infants were immunized with dissolved tablets or bacterial suspension measured with a syringe and added to tea.

Active protection test was carried out in mice with the mucin technique. For passive protection test 10-day chick embryos were used [1—3]. Equal amounts of sera from each mouse or infant group were mixed. Copro-antibodies were titrated in mice [1—3].

ED₅₀ values were estimated by Kärber's method [1—3].

Results

1. *Mouse experiments.* Groups of mice containing 4 animals each were fed graded doses of *E. coli* O111 Boivin antigen and bacterial suspension. The total doses were 2.0, 0.2, 0.02 and 0.002 mg Boivin antigen and 2×10^{11} , 2×10^{10} , 2×10^9 and 2×10^8 cells. In every group the corresponding total dose was given in 5 fractions at 3-day intervals. Five days after the last dose all the groups were challenged simultaneously with the homologous organism. The results were read 3 days after challenging and ED₅₀ values were estimated on the basis of the number of surviving/killed mice. The summarized results of 3 experiments are shown in Table I.

Table I

Protective effect in mice of E. coli O111 Boivin antigen and formalinized vaccine given in 5 doses at 3-day intervals

Challenging was performed by intraperitoneal mucin technique with *E. coli* O111

Experiment	Immunogenicity of antigens (ED ₅₀)			Challenging dose LD ₅₀
	Boivin antigen		Corpuscular vaccine, No. of bacteria	
	mg	equivalent No. of bacteria		
1	0.35	3.5 × 10 ¹⁰	4.4 × 10 ¹⁰	100
2	0.20	2 × 10 ¹⁰	4.5 × 10 ¹⁰	75
3	0.22	2.2 × 10 ¹⁰	3.8 × 10 ¹⁰	75

In all experiments the ED_{50} value against a challenging dose of 75–100 LD_{50} was represented by 0.2–0.35 mg Boivin antigen. To reach an identical degree of immunity, $3.8-4.4 \times 10^{10}$ bacterial cells were needed as corpuscular vaccine. Accordingly, these preliminary experiments showed that the protective effect of Boivin antigen was in correlation with the number of bacteria representing the Boivin antigen.

In subsequent experiments it was studied whether active immunity developing in mice treated with Boivin antigen or with equivalent numbers of bacteria can be confirmed in passive protection tests. Serum conversion in mice revaccinated with small doses was also studied (Table II).

Table II shows that there is a correlation between ED_{50} values expressed as the number of bacteria used for immunization and the amount of serum affording passive protection (RP). As in previous examinations [1], after revaccination with small amounts of vaccine the protective titres rose higher. Both Boivin and corpuscular antigens gave rise to copro-antibodies. Preliminary experiments definitely indicated that in immunizing power the bacterial suspension was equivalent to Boivin antigen.

The above experiments yielded a basis for a comparative study in infants of the effect of the two kinds of vaccine.

2. *Examinations in infants.* In the first experiment, 16 infants were immunized with polyvalent vaccine (O111, O55, O86). Groups of 4 were selected so that each contained individuals representing all the available age groups. A total dose of 9×10^{11} cells for each component of the corresponding amount of Boivin extract (9 mg for each antigen = 18 tablets) was given. The results obtained after vaccination at different intervals and after revaccination are shown in Table III.

In each group two kinds of inoculation interval were applied. Eight infants received 3 doses at 5-day intervals, while 8 infants 6 doses on 6 consecutive days (according to earlier experiments, continuous vaccination yielded somewhat better results). The findings were as follows.

(a) Boivin antigen and bacterial suspension given at 5-day intervals produced practically identical protective titres (RP = 5 and 3.2, respectively). Continuous vaccination with 6 doses was about twice as effective (RP = 10).

(b) Protective antibody titres were low and corresponded to titres obtained earlier with suboptimum doses of Boivin antigen [2, 3].

(c) Revaccinations resulted in a 2–3-fold increase in protective antibody titre (except one experiment where technical errors may have occurred). The raising of titres by revaccination is indicative of an insufficient basic immunization.

In view of these results, in subsequent experiments the immunizing effect of higher doses of antigen has been examined. As seen in Table IV, 4 groups of 3 infants each were subjected to continuous vaccination. Two

Table II

*Protective effect of E. coli 0111 Boivin antigen and formalinized bacteria in mice immunized orally with 5 doses at 3-day intervals, and passive immunity induced with the immunized animals' sera**

Antigens	Mode of immunization	Active immunity				Passive immunity					
		mg	No. of bacteria (ED ₅₀)	Challenging dose, LD ₅₀	Increase**	Serum, ml (RP)	Challenging dose, LD ₅₀	Increase**	Intestinal extract, RP/ml	Challenging dose, LD ₅₀	Increase**
Boivin	Basic 6 revaccinations	0.2	2 × 10 ¹⁰	75	10	0.023	50	6	0.013	20	6
		0.02	2 × 10 ⁹			0.004			0.002		
Bacterial suspension	Basic 6 revaccinations		3.5 × 10 ¹⁰	75	13	0.038	50	2.4	0.029	20	1.2
			1 × 10 ⁹			0.016			0.024		

* Mean values of 2 experiments

** Increase in protective effect between basic immunization and revaccination

Table III

Protective effect in infants of oral vaccination with *E. coli* O111 Boivin antigen and equivalent numbers of bacteria (9×10^{11})

Passive protection test in chick embryos; $LD_{50} = 25$ cells

Antigen	No. of vaccinations	Interval, days	Blood specimen taken	Dose and No. of bacteria				Protective effect of sera	
				Single dose*		Total dose*			
				No. of tablets (mg/antigen)	Equivalent No. of bacteria	No. of tablets (mg/antigen)	Equivalent No. of bacteria	ml	RP
Boivin	3	5	5 days after immunization after 6 revaccinations	6 (3)	3×10^{11}	18 (9)	9×10^{11}	0.009	5
				1 (0.5)	5×10^{10}	6 (3)	3×10^{11}	0.009	5
	6	1	5 days after immunization after 6 revaccinations	3 (1.5)	1.5×10^{11}	18 (9)	9×10^{11}	0.005	10
				1 (0.5)	5×10^{10}	6 (3)	3×10^{11}	0.0028	17
Bacterial suspension	3	5	5 days after immunization after 6 revaccinations		3×10^{11}		9×10^{11}	0.016	3.2
					5×10^{10}		3×10^{11}	0.005	10
	6	1	5 days after immunization after 6 revaccinations		1.5×10^{11}		9×10^{11}	0.005	10
					5×10^{10}		3×10^{11}	0.0016	32

The protective titre of infants' sera before immunization was 0.05 ml. This value represents the RP : 1 index

* For one component

Table IV

Protective effect in infants of oral vaccination with *E. coli* O111 Boivin antigen and equivalent numbers of bacteria (1.2×10^{12})
 Passive protection test in chick embryos: LD₅₀ = 25 cells

Antigen	No. of vaccinations	Interval, days	Blood specimen taken	Dose and No. of bacteria				Protective effect of sera	
				Single dose*		Total dose*			
				No. of tablets (mg/antigen)	Equivalent No. of bacteria	No. of tablets (mg/antigen)	Equivalent No. of bacteria	ml	RP
Boivin	6	1	5 days after immunization after 6 revaccinations	4 (2)	2×10^{11}	24 (12)	1.2×10^{12}	0.0009	55
				1 (0.5)	5×10^{10}	6 (3)	3×10^{11}	0.0009	55
	3	1	5 days after immunization after 6 revaccinations	8 (4)	4×10^{11}	24 (12)	1.2×10^{12}	0.0009	55
				1 (0.5)	5×10^{10}	6 (3)	3×10^{11}	0.0005	100
Bacterial suspension	6	1	5 days after immunization after 6 revaccinations		2×10^{11}		1.2×10^{12}	0.0016	31
					5×10^{10}		3×10^{11}	0.0016	31
	3	1	5 days after immunization after 6 revaccinations		4×10^{11}		1.2×10^{12}	0.0009	55
					5×10^{10}		3×10^{11}	0.0009	55

The protective titre of infants' sera before immunization was 0.05 ml. This value represents the RP : 1 index

* For one component

groups received 6 doses, and two groups 3 doses, on consecutive days. Each dose for the latter groups contained a double amount of Boivin antigen and cells as compared to doses given to the former groups. The total dose was 12 mg (24 tablets) for each Boivin antigen incorporated in the vaccine and 1.2×10^{12} bacteria for each component of the corpuscular vaccine.

From Table IV the following may be concluded.

(a) Immunization for 6 days was as effective as immunization for 3 days if the same total dose had been applied.

(b) There was no difference in immunogenicity between Boivin antigen and the equivalent bacterial vaccine.

(c) Revaccination did not increase the protective titre developing after the basic immunization. According to our earlier experience this result was characteristic of vaccination with optimum antigen doses. In both the present and previous [1, 2] studies, 24 tablets (12 mg for each antigen) of Boivin antigen were sufficient to give rise to a protective titre which was not increased for this by vaccinations.

(d) The total amount of antigen (36 mg Boivin antigen or 3.6×10^{12} cells for the 3 components) represented a large amount of endotoxin even if it was given in 3 fractions. Still, the infants showed no evidence of toxic symptoms.

In order to examine whether the bacterial suspensions exerted a polyvalent immunogenicity, the sera collected for the experiments shown in Table IV were examined for protective titre against the three vaccine strains. Results are listed in Table V.

Table V

*Polyvalent immunogenicity of vaccines given in 6 and in 3 doses**

Antigen	Vaccination, days	Blood specimen taken	RP values		
			O111	O55	O86
Boivin	6	5 days after immunization after 6 revaccinations	55	100	55
			55	100	55
	3	5 days after immunization after 6 revaccinations	55	100	100
			100	55	55
Bacterial suspension	6	5 days after immunization after 6 revaccinations	31	31	27
			31	31	55
	3	5 days after immunization after 6 revaccinations	55	31	31
			55	55	31

* Doses and amounts of antigens are shown in Table IV

Boivin antigen and bacterial suspension increased to the same extent as the protective titres to all strains incorporated in the vaccine. Revaccination failed to raise the titres further. Continuous vaccination for 6 days yielded the same result as 3 vaccinations with double doses.

Discussion

Our earlier immunizations of infants have been considered successful on the basis of serological examinations. In connection with these studies, two problems emerged, namely, is a simple corpuscular vaccine as effective as the Boivin antigen and, on the other hand, can the immunization period be shortened? The first problem is of an economic, the other of practical importance.

Four repeated experiments in mice vaccinated with Boivin antigen and with formalinized corpuscular vaccine have unequivocally confirmed that the degree of immunogenicity was associated with the germ equivalence of corpuscular vaccine and Boivin antigen. Revaccinations with corpuscular vaccine and with Boivin antigen stimulated the production of antibodies in a similar degree. Changes in antibody titres of mouse sera were identical after immunization with the two kinds of antigen. That the two vaccines exerted the same effect was indicated also by the fact that both of them gave rise to copro-antibodies.

The results were obtained with bacteria of the order of 10^{10} or with an equivalent amount of Boivin antigen. It is known that oral vaccination needs great amounts of antigen. Our doses were similar to those employed by other authors: MOCHMANN *et al.* [4] immunized mice with approximately 10^{10} cells and LIETZ and RAETTIG [5] used 9×10^9 bacteria. They immunized with a constant dose of antigen, challenged with an arbitrarily chosen number of bacteria and expressed the degree of immunity in percentage of survival. Our method of using graded amounts of immunizing antigen and a well-defined challenging dose (75–100 LD₅₀) allowed an exact determination in ED₅₀ of the efficacy of antigens.

After extraction, only about 70% of the original amount of antigen is contained in the Boivin antigen [6]. Accordingly, the Boivin antigen is in reality more effective than are bacterial cells. However, as equivalent bacterial amounts are inducing the same immunity, the corpuscular vaccine, which is less laborious to prepare and less expensive than Boivin antigen, is more advantageous for the purpose. The bacterial vaccine can be tabletted practically as well as the extract.

In our first studies on infants we used Boivin antigen slightly under the optimum dose (18 tablets = 9 mg antigen for each component) and an equivalent number (9×10^{11}) of bacteria as corpuscular vaccine. Although immunization at 5-day intervals as compared to daily vaccination for 6 days exerted a lower protective effect, both kinds of vaccine resulted in a 3–5-fold serum conversion. After revaccination of infants immunized at 7-day intervals the titres rose higher and reached a level appearing after continuous vaccination. With daily vaccination the protective titre was approximately twice as

high as with vaccination at 5-day intervals and revaccination resulted in a further increase. Thus the data indicated the higher efficiency of continuous vaccination and suggested the use of large doses.

On the basis of these findings a schedule yielding the best results was applied, namely, a total dose of 24 tablets (12 mg Boivin antigen for each component) or the equivalent number of bacteria (1.2×10^{12}) was given on 6 consecutive days (4 tablets or 2×10^{11} cells daily). Immunization reduced to a 3-day period with double daily doses (8 tablets or 4×10^{11} cells daily) was also tested. This schedule had yielded the best results ever experienced: there was a 30–55-fold rise in titre which did not increase after revaccination. This finding — corresponding to earlier observations — indicates that basic immunity reached with an optimum antigen dose cannot be increased further. It is noticeable that the 3-day immunization schedule was as effective as the 6-day one. Polyvalent immunity to all components of the vaccine (O111, O55 and O86) was also demonstrated. The number of bacteria (5×10^{10}) used for revaccination was equivalent to the booster dose of Boivin antigen (0.5 mg for each component).

As to the vaccines applied by other authors for the prevention of infantile enteritis, MOCHMANN *et al.* [4] gave 5 doses of desoxycholate extract each corresponding to 5×10^{10} cells. SCHREIBER [7] administered 7×10^{10} heat-killed cells. KÖDITZ *et al.* [8] used a single dose of 10^{12} streptomycin dependent cells. Our total dose contained, on the average, a number of bacteria 2 exponents higher than the doses of the above authors. However, the dose was not chosen arbitrarily but on the basis of experimental facts indicating that 24 tablets of Boivin antigen (12 mg of each antigen) or 1.2×10^{12} cells for each strain ensured the best results.

In the course of a nosocomial outbreak of *E. coli* enteritis, the vaccinated infants developed symptoms significantly less frequently than non-immunized ones [9]. In view of the data presented we believe that the time for a wide-scale trial of vaccination has arrived.

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AUSTRALIA ANTIGEN SURVEY IN HUNGARY*

I. HEALTHY BLOOD DONORS

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Summary. From the immunodiffusion (ID), immuno-electro-osmophoresis (IEOP) and complement-fixation (CF) tests, the IEOP test was found the most suitable for testing blood units before distribution. However, donors with subclinical hepatitis or those being in the incubation period may remain unrecognized by this test. The low Au-antigen titre and/or anti-complementary activity of the sera of some of these donors can be detected by the more time-consuming CF test.

Among 66 403 blood donors examined by the IEOP test, 662 Au-antigen carriers were detected. Antigenaemia was significantly more frequent in the male donors than in the female ones, and it was more frequent in young men than in those over 30 years of age. It is proposed that in these differences, besides genetic ones, hormonal factors may play a part. Age-dependence was less pronounced in the female donors. Antigen carriers were distributed in the ABO blood groups proportionally. Of 100 Au-antigen carriers followed up for a year, 92 remained positive.

The recognition of the close relation between serum hepatitis and the Australia (Au) antigen (SH antigen, HAA) [1, 2, 4—6] has made us to examine for Au antigen each unit of blood and blood preparation before use [7]. Methodical experience as well as the distribution of the positive donors by sex, age and blood group as compared to that of all the donors tested, will be discussed in the present report.

Materials and methods

Reagents. Au antigen and antibody preparations of human origin were used. Reference preparations were kindly supplied by Dr. A. M. PRINCE (New York) and Prof. J. L. MELNICK (Houston).

Our own antigen and antibody preparations were obtained from two patients with Down's syndrome and four patients suffering from haemophilia A, respectively. The identity of these reagents with the reference preparations was proved by the immunodiffusion (ID) and the complement-fixation (CF) tests (see below). Our preparations were not anticomplementary and gave no nonspecific reaction with Au-antigen-negative human sera.

Micro-ID test. The micro-Ouchterlony test was carried out as modified by PRINCE [8, 9]. One per cent agarose was dissolved in a solution containing 0.1 M NaCl, 0.01 M Tris buffer (pH 7.6 at 25°C), 1 g protamine sulphate, 0.001 M ethylene-diaminetetraacetic acid and 0.1 g merthiolate in 1 litre.

Some protamine sulphate preparations made the gel turbid without increasing the sharpness of the precipitation line. Such preparations were not used in screening tests.

Ten ml gel was poured into each Petri dish 10 cm in diameter, and four to six systems each consisting of a central well and six wells around it were prepared in each gel layer. The wells were 3 mm in diameter, the distance between the neighbouring wells was 4 mm. The

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Petri dishes were covered and incubated for 18–24 hours at 37°C and, subsequently, for 48 hours at room temperature. Results were read daily.

The IEOP test was carried out as described by KRASSNITZKY *et al.* [10]: 0.6% agarose was dissolved in a 0.13 M sodium barbiturate–sodium acetate buffer of pH 8.1. Nine ml gel was layered on a 5 × 18 cm glass plate and two paired rows of wells were prepared in each layer. The wells were 3 mm in diameter, the distance between the neighbouring wells was 4 mm. Forty serum samples including an antigen-positive control were tested in the 80 wells of each plate. The wells with antibody samples were placed on the anode side. The circuit was closed by filter paper stripes which were fixed on the gel by melted agarose gel. Voltage was kept between 90 and 110 V, the intensity of the current ranged between 8 and 10 mA/plate. After one-hour running, the plates were kept in a wet chamber at 37°C for another hour. To read the results, the precipitation lines were illuminated obliquely from below.

CF test. Sera were heated for 30 minutes at 56°C. The test was carried out on TAKÁTSY'S Microtiter plates [11]. Three rows of twofold dilution series were prepared from each serum, first dilution being 1 : 5 in row 1 and 1 : 2 in rows 2 and 3. Two units of guinea-pig complement were dropped into each well. Subsequently, 2 fixation units of antibody and antigen were added to each mixture in rows 1 and 2, respectively. To those in row 3, the same volume of buffer was added. The plates were kept in a wet chamber at +4°C overnight. Then a mixture containing 2.5% sheep erythrocytes in properly diluted rabbit haemolysin was added and the plates were incubated at 37°C for 2 hours. The highest dilution showing 50% or less lysis was taken as titre.

Routine laboratory tests for screening donors. Serum: bilirubin [12], SGPT [13], thymol turbidity and gold sol test [14]. Urine: urobilinogen and bilirubin (Rosin). In addition bromsulphalein retention was determined according to Selington in 100 chronic carriers.

Results

A total of 18 717 serum samples obtained from blood donors in the period from August to November, 1970, was examined by the ID test. As seen in Table I, 33 samples (0.18%) proved to be Au-antigen-positive.

It is a great disadvantage of the ID test that the results cannot be evaluated in the first 24 hours. Sometimes, reactions seemingly negative in the 24th hour became positive one or two days later. Consequently, the final result could not be awaited, especially in cases of rare blood-group combinations. For this reason, we tended to introduce the IEOP test. Before doing so, we re-examined in parallel with both tests 5000 serum samples of which 22 had been found positive, the others were selected at random from negative samples.

In the 24th hour, the repeated ID test revealed 20 positive sera; the 21st serum turned into positive on the second day, the 22nd serum on the third day. All the other sera remained negative. The IEOP test, on the other hand, showed 23 positive sera within 2½ hours. When in the 24th hour the former reactions were read repeatedly, no further positive reaction could be noticed.

Considering that in its technical performance the IEOP test is not more difficult than the ID test, we have exclusively applied the IEOP test in routine work since December, 1970.

Other investigators [15] found the micro-CF test 24 times more sensitive than the ID test for the detection of the Au antigen in human sera. It seemed therefore interesting to compare the more time-consuming CF test with the IEOP test as regards sensitivity in donor screening. For this purpose, 211

coded serum samples, including 28 positive and 183 negative samples as tested by the IEOP test, were submitted to the CF test.

CF antigen was demonstrable in all the 28 positive sera up to 1:80 or higher dilution. None of the sera were anticomplementary or contained anti-Au antibody. In the 183 negative samples neither Au antigen nor antibody could be detected by the CF test. One sample was anticomplementary up to the 1:4 dilution.

In the light of the present studies and literary data, we consider the time- and labour-saving IEOP test sufficiently sensitive for donor screening. The greater sensitivity of the CF test is valuable in testing sera with low Au-antigen titre or those containing anticomplementary antigen-antibody complex.

Table I
Donor screening for Au antigen

Test	Year	Month	Number of donors tested	Positive donors	
				No.	per cent
ID	1970	August	983	4	0.40
		September	4 430	11	0.25
		October	7 074	4	0.06
		November	6 230	14	0.22
		Total	18 717	33	0.18
IEOP	1970	December	5 503	40	0.73
	1971	January	5 283	22	0.42
		February	5 522	93	1.68
		March	6 018	59	0.98
		April	5 022	66	1.31
		May	6 079	79	1.30
		June	4 807	64	1.33
		July	4 287	43	1.00
		August	3 655	34	0.93
		September	5 328	44	0.83
		October	5 582	50	0.90
		November	4 525	32	0.71
		December	4 792	36	0.75
		Total	66 403	662	1.00

Table I shows the results of donor screening in monthly distribution. In the four months when the ID test was applied, the frequency of Au-antigen positivity ranged between 0.06 and 0.40% (mean, 0.18%). In the subsequent 13 months, 0.42–1.68% (mean, 1.00%) of the blood units examined by the IEOP test proved to be positive. The large difference in positivity between the first four and the subsequent 13 months cannot be explained by monthly fluctuations alone. It should mainly be attributed to the greater sensitivity of the IEOP test. Consequently, the results obtained by the ID test should be omitted in estimating the frequency of Au-positive blood units in our material. The 1.00% positivity, based on the data of 13 months, appears to reflect the real situation.

The exact distribution of the donors by sex and age was available for the months March, July, November and December, 1971 (Table II and Fig. 1).

Table II

Sex and age distribution of donors (March, July, November and December, 1971)

Sex	Au-antigen		Total
	negative	positive	
Females	4 980	25	5 005
Males	14 481	145	14 626
Total	19 461	170	19 631

$$\chi^2 = 10.5 \quad P < 0.01$$

In these months 25% of the blood donors and only 15% of those found positive for Au antigen were women. This implies that Au antigen was carried by relatively more male than female donors. A preliminary test of significance (χ^2) supported this statement. The 15% participation of females in the positive blood donors agrees well with the distribution by sex of the 695 donors found to be positive during the 17 months. Of these, 110 (16%) were women.

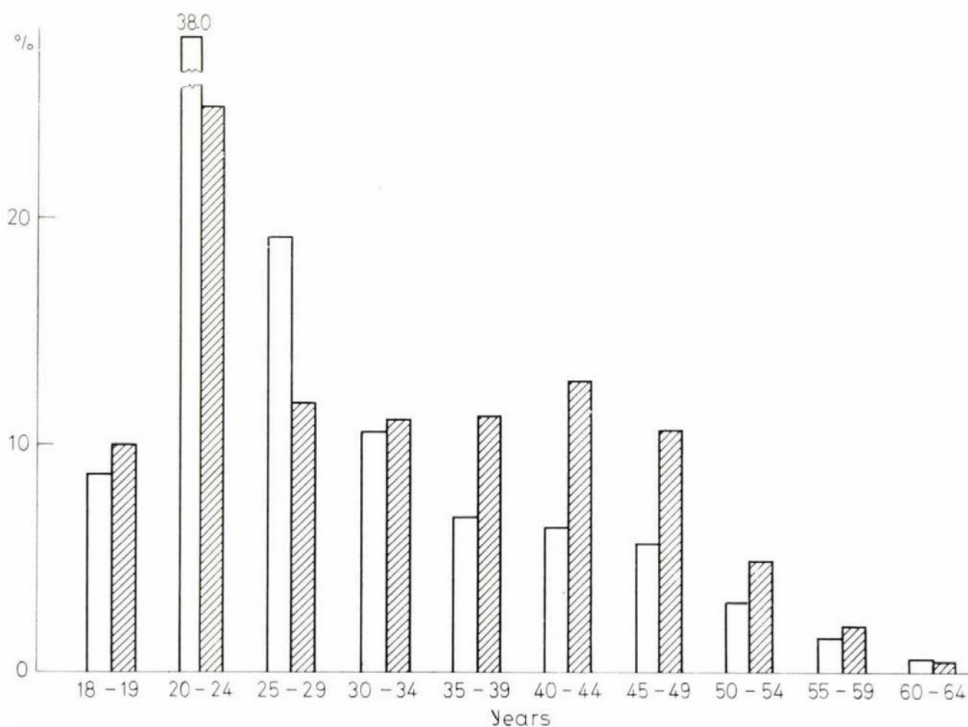


Fig. 1. Percentage distribution of male and female donors by age. Empty columns, male donors; hatched columns, female donors

The age distribution of the donors who were tested in the 4-month sample is given for five-year age groups in Fig. 1. The age limit of the donors is 18 years and 64 years. Within these limits the contribution of 20–24-year-old males was remarkably high. Among women the predominance of young donors was less pronounced. It was another difference in age distribution between the two sexes that the curve of the male donors was steadily declining while that of the females showed a plateau between 25 and 49 years.

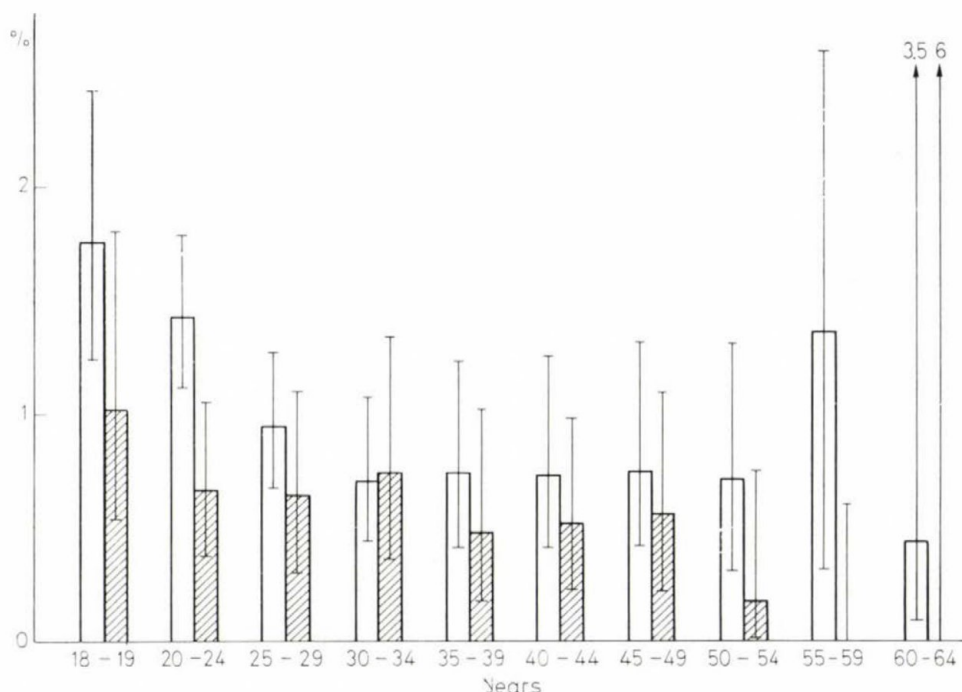


Fig. 2. Frequency of Au antigenaemia in male and female donors. Empty columns, male donors; hatched columns, female donors; I, fiducial limits (see text)

The validity of the age and sex distribution in the four-month sample was to be scrutinized. Considering that (a) the sample represented 30% of the whole material; (b) there was no discrepancy in the monthly sex and age distribution; (c) months belonging to all the four seasons were sampled; (d) no modification in the system of blood-transfusion service was introduced in the period under study, the validity of the sampled data seemed to be acceptable. Nevertheless, extra caution may be exerted. For this aim, allowance of $\pm 10\%$ fluctuation of the estimates for age and sex distribution of the donors examined during 13 months was made, i.e., the 0.95 fiducial limits computed according to the Poissonian law were broadened by 10%. The age-specific frequency is presented for five-year age groups in Fig. 2.

It is clear that the frequency of Au-antigen carriers was higher in the young male donors than in those over 30 years of age. The significance of this difference is clear. There is a plateau between 30 and 54 years, and age-specific frequency is again higher for the 55- to 59-year-old male donors. However, the significance of this second peak is reduced by the fact that only 10 positive donors belonged to this age group.

The age-specific frequency curve of female carriers starts at a relatively high level, shows a plateau at a lower level between 20 and 49, and a rapid fall after 50 years of age.

Returning to the sex distribution, the relative frequencies in all the 10 age groups but the 30-34 one proved to be higher for the males. The sign-test yielded $P = 0.02$, i.e., the relative frequency was significantly higher for the males. (As the 30-34 age groups did not differ much from the neighbouring one, this one exception out of 10 had to be considered an error of 2nd kind.)

To investigate the duration of carriership, 100 Au-antigen-positive donors (76 men and 24 women) were sampled late in 1970, and early in 1971, strictly in the order of arrival. These subjects were tested monthly for Au antigen for a year. Eight of them had become negative in 4-6 months, the others were still positive at the end of the observation period.

Seventy-two of the sampled positive donors remembered no event which might have related to hepatitis. Hepatitis had occurred within 5 years in the environment of 9 donors. Six had received blood transfusions in connection with a major operation, 11 remembered recent minor operations or injection treatment; two were chronic alcoholics.

None of the 100 donors became ill with hepatitis during the one-year observation period. Neither direct questioning nor clinical examination and laboratory tests revealed any symptom or sign suggestive of hepatic damage in the 72 donors with a negative history, nor in the 11 who had undergone some minor operation nor in 10 out of the others. Of the remaining 7 subjects, 6 had gastric complaints and hepatic tenderness to pressure, and one (C. C. P.) had epigastric pain and felt weakness over a period of a month. In this case, the physician's diagnosis was cholecystopathy.

Clinical examination and laboratory tests raised no suspicion of hepatitis except in 5 out of the 7 donors who had had complaints. C. C. P. had hepatomegaly, SGPT > 60 U and serum bilirubin levels between 1 and 2 mg/100 ml. This subject became Au-antigen-negative two months later. The other four, all of whom remained persistent carriers, had palpable liver, two of them had elevated SGPT and three had serum bilirubin between 1 and 2 mg/100 ml.

Apart from these, the laboratory tests listed under Materials and methods including the bromsulphalein-retention test proved to be negative for all the sampled donors. In the most suspect case (C. C. P.), Antweiler's serum-protein analysis was also carried out, and proved to be negative.

In conclusion, 93 of the 100 most persistently Au-positive donors proved to be healthy and at most 7 may have undergone abortive or sub-clinical hepatitis during the one-year observation period. This was most probable in the case of C. C. P., whose antigenaemia then soon ceased.

For analysis by blood group, the donors were not separated by sex because to our best knowledge the distribution by ABO groups is not sex-dependent.

Table III
Distribution of donors by blood group

Designation of donors		Distribution by blood group				Total
		A	B	O	AB	
Donors tested	No.	3 586	1 754	3 033	944	9 317
Nov. and Dec. 1971	per cent	38.5	18.8	32.6	10.1	100
Au-positive donors Nov. and Dec. 1971	No.	26	11	25	6	68
	per cent	38.2	16.1	36.8	8.8	100
Au-positive donors during 17 months	No.	278	142	204	71	695
	per cent	40.0	20.4	29.4	10.2	100

The data in Table III clearly show that the donors' Au-positivity was not influenced by their ABO blood group.

Discussion

The primary aim of our investigations has been to prevent Au-positive blood units and blood preparations from being transfused [16, 17]. The practical success of the screening cannot yet be estimated. It may be considered its first manifestation that the high anti-Au antibody titres of our haemophiliacs who used to serve as antibody sources is steadily declining, supposedly due to the lack of recent antigenic stimuli.

Like other investigators, we found the IEOP test the most suitable for the screening of donor bloods. We have confirmed that the CF antigen titre of the persistently Au-antigen-positive healthy persons is in general sufficiently high to be detected by the IEOP test [18]. Besides, the serum of these donors is usually not anticomplementary and contains no detectable amounts of anti-Au antibody. In subclinical cases of serum hepatitis, on the other hand, both the antigen titre and the anticomplementary activity may remain below the

sensitivity threshold of the IEOP system [18, 19]. For the detection of such cases, which amount to a small part of the Au-antigen-positive donors, the automated CF test [20] seems to be the most suitable.

The present work supplied information on the frequency of Au-antigen carriers in the healthy population of Hungary. The 1% frequency is not far from that found in other European countries. Although the monthly fluctuation of the frequency was high, a real seasonality seemed to be unlikely because the great majority of the positive donors remained positive at least for a year.

The difference between male and female donors in the frequency of Au-antigen positivity was unexpected. In this respect, the genetical determination suggested by BLUMBERG *et al.* [21] may be valid if it is supposed that due to endogenous and/or exogenous factors, the recessive gene manifests itself preferably in males. As an endogenous factor, the high hormonal activity might play a part in the high incidence of Au antigenaemia in young men. The persistence of the antigenaemia in the great majority of the regularly tested positive donors as well as the lack of anticomplementary activity and anti-Au antibody in the blood of the persistent carriers is consistent with the genetic hypothesis. In the presence of these factors one may think of subclinical illness, which however is rare if questioning, clinical examination and laboratory investigation of volunteers are made carefully.

It may be mentioned as a possible exogenous (environmental) factor that a large proportion of the young donors, especially the young males, were staying in colleges and semi-closed communities. In this respect, the high carrier frequency in subjects living under poor sanitary conditions [19] may be taken into consideration.

Another explanation of the age dependence of Au antigenaemia would be if persistent carriers were prone to developing hepatic damage or even hepatitis and thus were sooner or later lost as carriers. Of course, a spontaneous loss of the antigen which was carried for years cannot be excluded.

It seems therefore probable that several factors, *viz.*, genetic, sex, age, hormonal and environmental ones, play a part in the manifestation of Au antigenaemia.

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INVESTIGATIONS INTO THE OLIGOSACCHARIDE DECOMPOSITION OF SCHIZOSACCHAROMYCES OCTOSPORUS BEIJERINCK

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Summary. Investigating the oligosaccharide cleaving activity of *Schizosaccharomyces octosporus* against raffinose, sucrose and maltose it has been found that the strain only contains a maltose splitting enzyme, but this enzyme is different from the maltose cleaving enzymes of other species in that it shows extracellular activity in a suspension of intact cells.

During the study of the comparative physiology of yeasts we have investigated the connections between sucrose and maltose decomposition of several species [4, 5, 7—12]. On the basis of these results, two types of sucrose and maltose splitting enzymes have been distinguished. One of the sucrose splitting enzymes was found to be invertase [3, 4, 6, 13], while the other an intracellularly located, lipid solvent sensitive, sucrose specific enzyme [5, 7—12]. The maltose splitting enzymes were not closer investigated by us, although one of them was shown to be located intracellularly, resistant to lipid solvents, and not cleaving sucrose [3—5, 8, 9, 11, 12]. Consequently, this enzyme is not identical with yeast-maltase, provided the view is accepted that the typical yeast-maltases are able to cleave sucrose [2]. The other maltose splitting enzyme was observed by us in one single case; as much as it could be established, it was located intracellularly and was sensitive to lipid solvents [1].

On the basis of the above findings it seemed necessary to investigate the maltose splitting enzyme of a species not producing any sucrose splitting enzyme, as has been made by us in the opposite sense in a study of the sucrose decomposition by *Candida reequinii* [7]. Therefore, for the present investigation *Schizosaccharomyces octosporus* was chosen, a species assimilating and fermenting glucose and maltose only.

Materials and methods

In the experiments, a strain of *S. octosporus* of our collection was used; in view of its unknown origin, its identity was checked. The strain was cultivated on CSILLAG's molasses agar [1] in Roux flasks. The methods used in the study of living and acetone treated cells and cell free extracts as well as the applied technique of paper chromatography have been described [3, 4, 7].

Results

Raffinose cleavage. All the three preparations proved to be inactive against raffinose (Figs 1–3).

Maltose cleavage. Living cells cleaved maltose extracellularly (Fig. 4); acetone treated cells and cell free extracts also cleaved this sugar (Figs 5 and 6).

Sucrose cleavage. Living cells neither take up nor split sucrose and acetone treated cells and the cell free extract also failed to split this sugar (Figs 7–9).

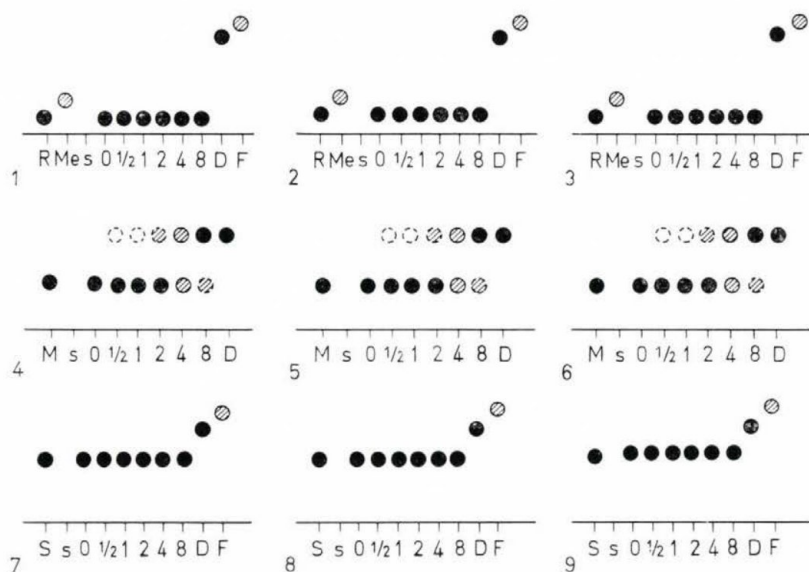


Fig. 1. Raffinose utilization by intact *S. octosporus* cells; 750 mg live wet cells and 60 mg raffinose in M/30 phosphate buffer pH 7.2 in 3 ml volume. Chromatogram. Left, raffinose, melibiose and suspension; right, glucose and fructose controls. Numbers under the start line represent sampling intervals in hours

Fig. 2. Raffinose utilization by acetone treated *S. octosporus* cells. Acetone treated 750 mg live wet cells. Others, as indicated in Fig. 1

Fig. 3. Raffinose utilization by cell-free extract of *S. octosporus* cells. Cell-free extract of quartz sand-disintegrated 750 mg live wet cells. Others, as indicated in Fig. 1

Fig. 4. Maltose utilization by intact *S. octosporus* cells. 750 mg live wet cells and 60 mg maltose in M/30 phosphate buffer, in 3 ml volume. Chromatogram. Left, maltose and suspension; right, glucose controls. Numbers under the start line represent sampling intervals in hours

Fig. 5. Maltose utilization by acetone treated *S. octosporus* cells. Acetone treated 750 mg live wet cells. Others, as indicated in Fig. 4

Fig. 6. Maltose utilization by cell-free extract of *S. octosporus* cells. Cell-free extract of quartz sand-disintegrated 750 mg live wet cells. Others, as indicated in Fig. 4

Fig. 7. Sucrose utilization by intact *S. octosporus* cells. 750 mg live wet cells and 60 mg sucrose in M/30 phosphate buffer pH 7.2 in 3 ml volume. Chromatogram. Left, sucrose and suspension; right, glucose and fructose controls. Numbers under the start line represent sampling intervals in hours

Fig. 8. Sucrose utilization by acetone treated *S. octosporus* cells. Acetone treated 750 mg live wet cells. Others, as indicated in Fig. 7

Fig. 9. Sucrose utilization by cell-free extract of *S. octosporus* cells. Cell-free extract of quartz sand-disintegrated 750 mg live wet cells. Others, as indicated in Fig. 7

Discussion

In contrast to our expectation, maltose was split by living cells extracellularly, *i.e.* in the incubation fluid the glucose concentration increased parallel with time. In the case of the appearance in the incubation mixture of a decomposition product, in the present case of glucose, two explanations may be proposed. The first one is that the splitting enzyme acts extracellularly — *i.e.* the enzyme is released by the cells or else it is localized on their outer surface — and thus the oligosaccharide will be split without permeation (transportation). The second supposition is that the oligosaccharide molecule permeates without being split, it is then split intracellularly and the cleavage product (monosaccharide) passes into the incubation mixture by re-permeation. The first assumption is contradicted by the fact that up to now no extracellular maltose cleaving yeast-enzyme has been found, but other enzymes, *e.g.* invertase, are known to split sucrose extracellularly. The second supposition is considerably weakened by the fact that metabolizable materials are not released by the cells under physiological conditions and such a monosaccharide re-permeation from the cells of yeasts cleaving oligosaccharides intracellularly has never been observed by us except after acetone treatment.

On the basis of the above, the first explanation seems to be correct, and thus *S. octosporus* has a maltose splitting enzyme differing from those isolated by us from other yeasts. The enzyme is resistant to acetone treatment and does not cleave sucrose.

Next it was shown that the investigated strain of *S. octosporus* does not contain invertase or any other sucrose cleaving enzyme.

Thus, we have succeeded in obtaining such an experimental object which in the absence of sucrose splitting activity would be suitable for the study of maltose decomposition although, on the basis of the present results, not in the case of the aforementioned two types of maltose cleaving enzyme, but in the case of a new, third type of enzyme.

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THEOPHYLLINE INHIBITS THE TRANSCRIPTION OF THE LAC OPERON IN ESCHERICHIA COLI

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Summary. Theophylline decreases the rate of transcription of the *lac* operon in inducible wild type *Escherichia coli* K 12 strain. This inhibitory effect is partly reversed by cyclic adenosine-3',5'-monophosphate. The translational process is not affected. The similarity of the effect of theophylline and that of glucose is apparent. The hypothesis is raised of a competition between theophylline and cyclic adenosine-3',5'-monophosphate in that system in which this latter promotes initiation of messenger ribonucleic acid synthesis on the *lac* deoxyribonucleic acid of *E. coli*.

In the mammalian organism the inhibition of specific cyclic adenosine-3',5'-monophosphate (cAMP) phosphodiesterase activity by theophylline was demonstrated by BUTCHER and SUTHERLAND [1]. This effect resulted in an increased intracellular concentration of cAMP. In contrast, in the *Escherichia coli* K 12 wild type strain we found a decrease of the synthesis of induced β -galactosidase in the presence of theophylline at concentrations of 3×10^{-3} M and higher [2]. The kinetics of the inhibitory action of theophylline reminded us of catabolite repression by glucose. cAMP — which is known to prevent the catabolite repression of the *lac* operon in *E. coli* [3, 4] — was able to reverse the inhibitory effect of theophylline. In explaining the mode of action of theophylline our findings and the well known role of cAMP in the transcription of the *lac* operon in *E. coli* [5] led us to suppose the inhibition of adenyl cyclase (the enzyme which forms cAMP from ATP) or a competition between cAMP and theophylline in that system in which cAMP promotes the transcription of the *lac* operon. Both these mechanisms may result in a decreased rate of the *lac* mRNA synthesis without inhibiting the translational process, according to the data of JAQUET and KEPES [6].

In the following we shall report on results concerning the mechanism of action of theophylline.

Materials and methods

The *E. coli* K 12 wild type strain was used throughout. An overnight culture of the cells in the following liquid medium was diluted in fresh, prewarmed medium and let to grow in a reciprocal shaker for an additional 1.5 hour in the log phase. The composition of the medium was NH_4Cl , 2.0 g; Na_2HPO_4 , 6.0 g; KH_2PO_4 , 3.0 g; NaCl , 3.0 g; MgCl_2 , 0.04 g; Na_2SO_4 , 0.116 g; Na-succinate, 10.0 g; casamino acids (vitamin-free, Serva, Heidelberg), 1.0 g per litre; pH 7.4. At an $\text{OD}_{570} = 0.300$ the culture was cooled in an ice bath and centrifuged in a refrigerated centrifuge at 3000 g for 20 minutes. The supernatant was discarded and the cells resuspended

in half amount of fresh medium of the same composition as above, but lacking the two carbon sources. The suspension was then shaken reciprocally for 30 minutes. After this starving period the experiment was started at an $OD_{570} = 0.600$.

To measure the rate of transcription, the method of JACQUET and KEPES [6] was used modified as follows: 4.0 ml aliquots of the above suspension were added at 0 min. to 1.0 ml of the medium (without carbon sources) containing isopropyl- β -D-thio galactoside (IPTG, Mann Research) to give a final concentration of 10^{-4} M for induction of β -galactosidase synthesis. Theophylline (pure, 5th Hungarian Pharmacopoeia) and/or cAMP (Sigma Chemical Co., St. Louis) was added to yield final concentrations of 10^{-2} M and 10^{-3} M, respectively. In the control experiments there were no additions except IPTG.

Induction of β -galactosidase was stopped by o-nitrophenyl- β -D-fucoside (ONPF, Sigma), the competitive antagonist of IPTG. At 1.5 minute of the induction, aliquots of 1.0 ml were taken from the induced suspension and pipetted into 0.5 ml of the medium (without carbon sources) containing ONPF to give a final concentration of 5×10^{-3} M. Translation of the *lac* mRNA produced during the induction period was allowed for an additional period of about 25 minutes. Samples of 0.1 ml were taken at intervals from the inhibited and from the non-inhibited suspensions and pipetted into test tubes standing in ice bath and containing 0.1 ml phosphate buffer pH 7.0 with cetyl-trimethyl-ammonium bromide (CTAB, Fluka), 1.0 mg/ml and chloramphenicol (EGYT, Budapest), 0.5 mg/ml. These samples were kept in the ice bath until β -galactosidase activity was estimated.

When the effect of theophylline upon translation was tested, the procedure was essentially the same, except that the substance was not present during the induction period but was added together with ONPF.

In other experiments the induced β -galactosidase synthesis was followed in parallel with the incorporation of (14 C)-L-leucine. In these experiments M 63 mineral salts minimal medium was used, 0.5 ml/100 ml glycerol served as carbon source. An overnight culture was diluted in fresh, prewarmed medium to an $OD_{570} = 0.075$. The cells were shaken until the density had reached an $OD_{570} = 0.300$. At this point the experiment was started by the addition of methylthio- β -D-galactoside (TMG, Sigma) to give a final concentration of 5×10^{-4} M. (14 C)-L-leucine (UVVVR Prague, spec. act. 100 μ Ci/ μ mole) was added at $OD_{570} = 0.150$ to give a final concentration of 0.4 μ Ci/ml. Samples for the determination of β -galactosidase activity were taken as described above. Samples of 1.0 ml were taken for the estimation of incorporated radioactivity and pipetted into 1.0 ml 10% ice cold trichloroacetic acid (TCA) solution. The precipitate was collected onto membrane filters (VCHZ Synthesia Synpor 6, pore diameter 0.4 μ), which were washed with 5% ice cold TCA and placed into scintillation vials. The 10 ml scintillation fluid per vial was composed of toluene, PPO and POPOP and counts per minute were read in a Packard Tri-Carb liquid scintillation counter.

β -Galactosidase activity was determined by the hydrolysis of o-nitrophenol- β -D-galactoside (ONPG, Fluka), as described earlier [2], and expressed as 1 unit = 1 μ mole ONPG hydrolyzed per 1 ml bacterial suspension per hour.

All the experimental procedures, with the above exceptions, were performed at 37°C.

Theophylline did not affect β -galactosidase itself in the assay mixture at the concentrations used in these experiments.

Results

In previous experiments [2] it remained open whether theophylline was able to decrease the induced β -galactosidase synthesis by inhibiting the growth rate of *E. coli* cells. In the first experiment we have therefore tested β -galactosidase synthesis and protein synthesis on the basis of (14 C)-leucine incorporation. Fig. 1 shows the differential rate of enzyme synthesis plotted against the incorporated radioactivity. Theophylline decreased protein synthesis, but the inhibition of β -galactosidase synthesis was more marked: the plot shows the typical picture of transient repression. This repression was, however, not so intensive as that caused by 10^{-3} M glucose, which is also shown in Fig. 1. cAMP at 10^{-3} M concentration decreased the repression caused by 5×10^{-3} M theophylline.

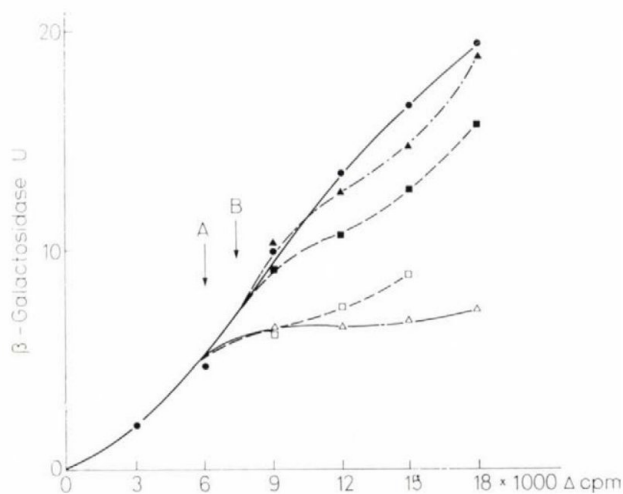


Fig. 1. Differential rate of β -galactosidase synthesis. An overnight culture of *E. coli* K 12 cells was diluted in fresh, prewarmed M 63 mineral salts minimal medium (supplemented with 0.5 ml glycerol and 2 mg L-leucine per 100 ml medium) to an $OD_{570} = 0.075$ and agitated in a reciprocal shaker at 37°C . When the cell density had reached $OD_{570} = 0.300$ the culture was induced with 5×10^{-4} M TMG (0 minute). At 15 minutes (arrow A) aliquots of this control culture were pipetted into Erlenmeyer flasks containing theophylline (10^{-2} M final concentration) or glucose (10^{-3} M final concentration). At 25 minutes (arrow B) aliquots of the induced culture were pipetted to theophylline (5×10^{-3} M final concentration) and theophylline + cAMP (5×10^{-3} M and 10^{-3} M, respectively). Shaking was continued; 1.0 ml aliquots were taken at intervals and pipetted to test tubes containing 0.1 ml solution of CTAB and chloramphenicol (1.0 mg/ml and 0.5 mg/ml, respectively). After vigorous mixing the samples were placed into an ice bath. β -galactosidase activity was estimated on the basis of ONPG hydrolysis: 1.5 ml of a 1.0 mg/ml solution was given to the samples and incubated at 37°C for 10 minutes. Hydrolysis was terminated by adding 1.0 ml of 1.0 M Na_2CO_3 . The absorbance of the supernatant was read at 420 m μ . (^{14}C)-L-leucine was added at an $OD_{570} = 0.150$ to give a final concentration of 0.4 $\mu\text{Ci/ml}$; 1.0 ml samples were taken and pipetted into 1.0 ml 10% ice cold TCA solution. The precipitate was collected onto membrane filters, washed with 5% ice cold TCA. The incorporated radioactivity was estimated in a Packard Tri-Carb liquid scintillation counter and expressed in counts per minute. Control $\bullet$ — $\bullet$; 10^{-2} M theophylline $\square$ — $\square$; 10^{-3} M glucose $\triangle$ — $\triangle$; 5×10^{-3} M theophylline $\blacksquare$ — $\blacksquare$; 5×10^{-3} M theophylline; 10^{-3} M cAMP $\blacktriangle$ — $\blacktriangle$

Table I
Rate of β -galactosidase synthesis

	$\frac{\Delta E_{420}}{\Delta \text{cpm}}$
Control	3.37×10^{-5}
Theophylline 10^{-2} M	1.25×10^{-5}
Glucose 10^{-3} M	0.53×10^{-5}
Theophylline 5×10^{-3} M	1.96×10^{-5}
Theophylline 5×10^{-3} M + cAMP 10^{-3} M	2.55×10^{-5}

Table I summarizes the rate of specific synthesis of β -galactosidase in the presence of various added substances. Factors which bring about decreased

growth of *E. coli* cells are known to cause catabolite repression through a weak carbon source in the medium, which evokes no repression by the normal growth rate [7, 8]. In preliminary experiments it was shown that theophylline decreases induced β -galactosidase synthesis in different media containing succinate, succinate + casamino acids or glycerol as carbon source as well as in a medium lacking any carbon source. Therefore, to avoid the above possibility, carbon-starved cells were used in the next experiment. Fig. 2 dem-

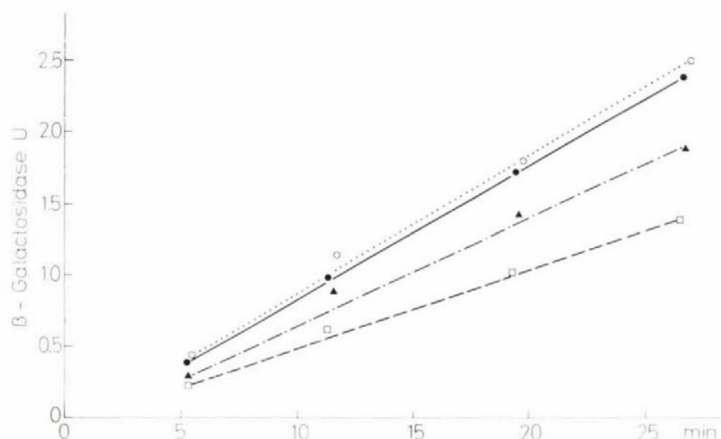


Fig. 2. Effect of theophylline and cAMP in carbon starving cells. Log phase cells were centrifuged, resuspended in medium lacking carbon source and agitated at 37°C in a reciprocal shaker for 30 minutes. After this period of starvation the cells were induced with 10^{-4} M IPTG and theophylline (10^{-2} M final concentration), cAMP (10^{-3} M), and theophylline + cAMP (10^{-2} M and 10^{-3} M, respectively) were added at 0 minute of the induction. Samples of 0.1 ml were taken and β -galactosidase activity was measured as described in Fig. 1. Control ●—●; theophylline □---□; cAMP ○····○; theophylline + cAMP ▲-·-▲.

onstrates the effect of theophylline upon induced β -galactosidase synthesis in this system. Theophylline at 10^{-2} M caused a marked inhibition of enzyme synthesis, to about 58% of the control. cAMP at 10^{-3} M concentration partly reversed this effect of theophylline (80% of the control). cAMP alone at the same concentration did not increase enzyme synthesis (101% of the control). The reversibility of the theophylline inhibition by cAMP gives rise to the hypothesis that theophylline may act at a transcriptional level.

To verify this hypothesis, the following experiment was carried out (Fig. 3). Induction of β -galactosidase was suspended by ONPF, the competitive antagonist of IPTG, at 1.5 minute of induction. In the control experiment, enzyme synthesis proceeded for some minutes, because ONPF stops the transcription by allowing the restitution of the operator gene—repressor protein complex, but does not interfere with the translation of mRNA molecules produced during the induction period [6] (= "pulse induction technique").

The mRNA molecules coding for β -galactosidase being exhausted, the rate of enzyme synthesis becomes lower and lower and at about the 18th minute of the experiment the curve of the synthesis levels off, showing that after this time no new enzyme has been synthesized. The level at which the enzyme content reaches its maximum and becomes horizontal is proportional to the amount of mRNA molecules synthesized on the *lac* operon during the period of induction. In the presence of 10^{-2} M theophylline, mRNA synthesis was

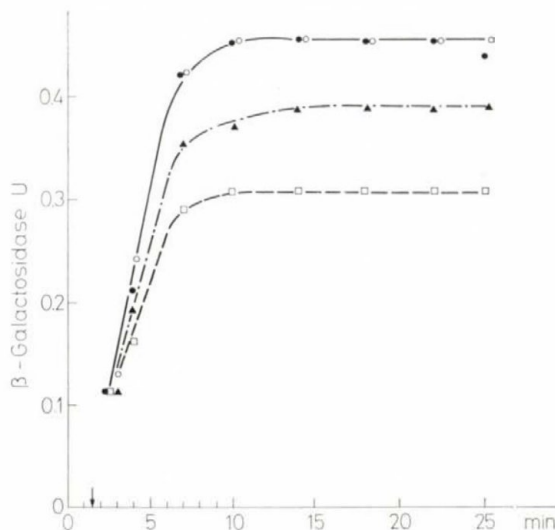


Fig. 3. Effect of theophylline and cAMP upon transcription of the *lac* operon. Carbon starving cells were induced at 0 minute with 10^{-4} M IPTG. The induced synthesis of β -galactosidase was terminated by adding 5×10^{-3} M ONPF at 1.5 minute (indicated by an arrow); 0.1 ml samples were taken at intervals and analysed for β -galactosidase activity in essentially the same way as described in Fig. 1. Additions were given at 0 minute as follows: theophylline (10^{-2} M), cAMP (10^{-3} M), theophylline (10^{-2} M) + cAMP (10^{-3} M). Control ●—●, theophylline □---□, cAMP ○---○, theophylline + cAMP ▲---▲

inhibited by 32% as compared to the control. If at 0 minute theophylline was added to the cells at the same concentration but with 10^{-3} M cAMP, this latter decreased the inhibition from 32% to 14%. cAMP alone did not increase the rate of transcription. If theophylline was not administered during the induction period but was added together with ONPF in the period of translation, no decrease of the enzyme level was seen in comparison to the control (not shown in the Figure). These findings support the hypothesis that theophylline inhibits β -galactosidase synthesis at the level of transcription.

Discussion

In *E. coli* — as well as in other bacteria — cAMP plays an important role as it was summarized by PASTAN and PERLMAN [9]. Among others, the transcription of the *lac* operon needs a physiological intracellular concentration of cAMP. The intracellular level of cAMP is influenced by (a) the adenylyl cyclase activity, which was detected by IDE [10]; and (b) the specific cAMP phosphodiesterase, which hydrolyzes cAMP to 5'AMP [11]. A third possibility was described by MAKMAN and SUTHERLAND [12] who found that in glucose containing medium *E. coli* cells had a lower intracellular level of cAMP and this became detectable in the medium. If the intracellular concentration of cAMP falls, the rate of transcription on the *lac* operon decreases. cAMP promotes the transcription of this operon by maintaining the RNA-polymerase binding property of the promoter region. According to EMMER *et al.* [13] and DECROMBRUGGHE *et al.* [14], cAMP is not bound directly to the DNA but acts only in the presence of a specific cAMP binding protein (CRP).

Glucose and other carbohydrates which cause a rapid lowering of intracellular cAMP concentration [12] cause a transient and catabolite repression of induced β -galactosidase synthesis in *E. coli* by decreasing the rate of transcription of the *lac* operon [6]. This effect can be counteracted by adding cAMP to the medium [9]. If theophylline would have the same effect in *E. coli* as it has in the mammalian organism [1], its effect would result in an enhanced *lac* mRNA production and in an increased rate of β -galactosidase synthesis, or — in the case of maximum synthesis of this enzyme — it would be ineffective. PASTAN and PERLMAN [9] were not able to show a possible effect of theophylline upon cAMP phosphodiesterase.

In a previous work [2] we have succeeded in demonstrating an inhibitory effect of theophylline upon induced β -galactosidase synthesis in our *E. coli* K 12 wild type strain, which reminded us of the transient repression caused by glucose or other carbohydrates. cAMP seemed to be effective against theophylline inhibition in these experiments, like in the case of glucose repression. It remained to be decided whether theophylline was able to decrease β -galactosidase synthesis by inhibiting the growth rate of the cells.

We were able to show that β -galactosidase synthesis was repressed to a greater extent than was overall protein synthesis as measured by the incorporation of (¹⁴C)-leucine into 5% ice cold TCA precipitable fraction. This finding led us to assume that theophylline had a specific decreasing effect upon induced β -galactosidase synthesis.

To avoid the error caused by a possible catabolite repression at the altered overall protein synthesis, experiments were done with carbon-starving cells. In this case no carbon source of the medium could be made responsible for a non-specific transient or catabolite repression at a decreased growth rate

of the organism. In these experiments theophylline was effective when present in the period of induction, *i.e.* of transcription, but did not affect the final β -galactosidase content administered after the *lac* mRNA synthesis had ceased, *i.e.* the translational process. However, cAMP decreased the inhibition caused by theophylline and has made us to assume a competition between theophylline and cAMP in the system.

SHEPPARD [15] showed theophylline to be an inhibitor of the adenyl cyclase activity of rat erythrocyte ghosts. In a bacterial system, the adenyl cyclase of *Nocardia erythropolis* was studied by IDE [16]; the enzyme was intensely inhibited by GTP, UTP, CTP, ITP, 3'-AMP, oxalacetate and pyridoxal phosphate. These findings suggest that, by inhibiting adenyl cyclase activity, theophylline lowers the intracellular cAMP concentration in *E. coli*. We cannot exclude this possibility, but it is not reasonable to consider this inhibition to represent the first point of attack of theophylline, its effect being instantaneous. It seems more probable that theophylline may compete with cAMP in that system, in which the latter compound promotes the transcription of the *lac* operon. To test this hypothesis, it is necessary to determine the quantitative relationships of theophylline and cAMP in the assumed competition and, also, whether theophylline is able to compete with cAMP for CRP.

Recently, ABOUND and BURGER [17] have succeeded in showing theophylline to be effective against catabolite repression caused by glucose. In one of their *E. coli* strains, theophylline inhibited cAMP phosphodiesterase activity and this resulted in an enhanced β -galactosidase synthesis in glucose and cAMP-treated cells in the presence of 10^{-3} M theophylline. This finding does not agree with the results of BRANÁ and CHYTIL [11] and PASTAN and PERLMAN [9], nor does it support our present data. The apparent contradiction may be based on the different genetic character of the different bacterial strains.

Most recent results in our laboratory are indicating the effect of theophylline to be not highly specific, chemically related and similar compounds being also effective. Investigations into the identity or differences in the effect of these substances are in progress.

Acknowledgement. We are indebted to Dr. J. JANAČEK for helpful discussions as well as for the generous gift of membrane filters and labelled leucine; to Dr. J. CSONGOR for radioactivity measurement facilities; to Mrs. I. SZEKERES for skilled technical assistance and to Miss I. MAGYAR and Mr. F. FÁBIÁN for the drawings.

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IMMUNOFLUORESCENCE STUDIES ON TISSUE CULTURES DOUBLY INFECTED WITH ADENO AND HERPESVIRUSES

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Summary. Experiments were carried out to establish whether adeno and herpesviruses were able to penetrate into, and produce antigens in, different cells of the same cell culture or even in the same cell. Using optimum time for infection with the two virus species — considering the different periods required for their replication — antigens of both virus species were detectable in the same tissue culture, frequently even within the same cell. The immunofluorescence technique was used, applying fluorescein isothiocyanate-conjugated adenovirus antisera and rhodamine-conjugated herpesvirus antisera. Antigens could be differentiated on the basis of their localization and according to the colour of the conjugates. The findings have confirmed our earlier observations made on specimens taken directly from patients.

A wide range of studies has been published on the interaction of different viruses, on their enhancing or hindering effect on replication [1, 13, 21, 22].

It has been observed in previous experiments that infective adenoviruses and herpesviruses may be present simultaneously in the epithelial cells of patients with oral ulcers. The finding has been confirmed by virus isolation [14]. Two kinds of viral antigen were found in a significant percentage of the oral mucosa cells of such patients. Moreover, the simultaneous occurrence of the two antigens was detected within the same cell [15] in some cases.

On the basis of these results the question arose of the behaviour of two viruses being present simultaneously in the same tissue culture. Studies of this problem will be reported in the present paper.

Materials and methods

Adenovirus type 1 and *Herpesvirus hominis* isolated in our laboratory from patients with oral ulcers have been used in the experiments. Primary human amnion cultures were prepared by trypsinization in test tubes containing cleaned and sterilized coverslip fragments 6 × 18 mm in size, and used for infection. The culture medium consisted of Hanks' solution containing 10% human serum and 0.04% lactalbumin hydrolysate, the maintenance solution applied after infection was composed of 67% Hanks' solution, 30% Parker's 199 solution and 3% calf serum. The usual antibiotics were added to the culturing and maintenance media.

Three to four weeks old tissue cultures were infected by placing 100 CPU of adenovirus type 1 on the coverslip cultures, previously washed with Hanks' solution. Three to four days later when 25 to 30% of the cells showed signs of viral effect, the cultures were superinfected with 100 to 200 CPU of *Herpesvirus hominis*. Sixteen to eighteen hours after the second infection the coverslip fragments were washed four times with physiological saline and fixed in methanol.

Uninfected amnion cultures prepared and treated identically as well as tissue cultures infected with adenovirus or with herpesvirus were used as control.

Immunofluorescence examinations were performed by the direct method of COONS and KAPLAN [16]. Hyperimmune sera against the viruses were prepared in rabbits using virus pools pre-treated with Freund's adjuvant. The first two injections were given intramuscularly at a ten-day interval, the further 4 injections were administered intravenously at intervals of 3 to 4 days. The following technique was used for conjugation of the sera: globulin fractions of sera prepared against adenovirus type 1 or herpesvirus were precipitated three times with semi-saturated ammonium sulphate. Concentrated and dialysed globulin fractions of adenovirus type 1 antiserum were conjugated with fluorescein isothiocyanate using 1 mg stain to 120 mg protein, the S : P ratio being thus 3 : 1. Globulin fractions of herpesvirus antiserum were conjugated with Lissamine-Rhodamine B-200, adding 10 mg fluorochrome to each 100 mg of protein. A 10% solution of fluorochrome was applied, the solvent being dry acetone. Both conjugates were subjected to purification by Sephadex G-25 column chromatography and treated with merthiolate.

Staining was carried out with both conjugated sera simultaneously or successively. In the case of simultaneous staining the conjugates of the mixed sera were used in 1 : 2 dilution, buffered physiological saline being used as diluent. Preparations were covered for one hour with the conjugates, then washed for one hour in physiological saline, changing the solution three times, subsequently rinsed with distilled water and dried, finally evaluated under a Zeiss microscope ($\times 600$) using an UG—3.5 exciter filter and a GG9 barrier filter.

Results

A yellow-green fluorescence, corresponding to the viral antigen and to the conjugate, was found in the cells infected with adenovirus, while an orange-red fluorescence in those infected with herpesvirus. The orange-red fluorescence appeared first in the nucleus and later in the cytoplasm. It is known that with the two virus species different periods are required for the development of viral antigen and for the appearance of a cytopathic effect. Thus, to obtain a fluorescence of identical intensity at one given period it was necessary to determine the optimum time of infection. In the case of simultaneous infection, the herpesvirus antigen, developing earlier, showed an intensive fluorescence at a time when the adenovirus antigen revealed a slight or

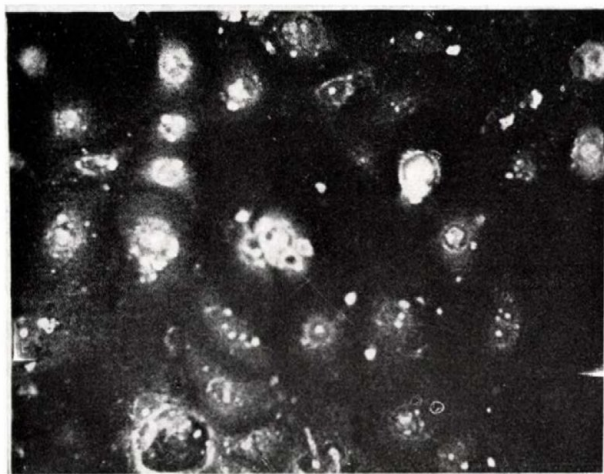


Fig. 1



Fig. 2

no fluorescence. To obtain a fluorescence of identical intensity, infection with adenovirus had to precede by 72 hours that with herpesvirus. Pre-infection with adenovirus did not inhibit the development of herpesvirus antigen, only the time of its appearance was delayed. Specific fluorescence referring to both viral antigens could be detected in doubly infected tissue cultures, using the same method. Certain cells of the preparations showed fluorescence characteristic of adenoviruses, while others displayed typical herpesvirus fluorescence. A certain percent of the cells was found to contain both viral antigens. In the case of double infection the yellow-green adenovirus fluorescence was observed in the nucleus, while the orange-red herpesvirus fluorescence in the cytoplasm or directly beside the nuclear membrane, occurring often in small clots (Fig. 1).

The uninfected cultures, subjected to identical treatment, revealed only auto-fluorescence (Fig. 2).

Discussion

Both adenoviruses and herpesviruses have been widely studied by the immunofluorescence method [17–19]. The method was suitable to determine, among others, the time of appearance of specific viral antigens, their localization, etc., in different tissue cultures. The simultaneous effect of herpes and some other viruses had also been studied [20].

Studies concerning the ability of adeno and herpesviruses to multiply in each other's presence or to produce antigens in the same tissue culture have not been found in the available literature. Our experiments revealed that antigens of both virus types may develop in different cells of the same tissue culture, in some cases even within the same cell, confirming thus our earlier findings in specimens taken directly from patients [15]. The two antigens are

easy to differentiate on the basis of their location and the colour of the conjugates. The correlation between this phenomenon and the clinical manifestation has not been cleared. However, the fact that it is infrequent in healthy individuals refers to a possible common role of the two viruses in certain clinical conditions.

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CHROMATOGRAPHISCHE POLIO-ANTIKÖRPERBESTIMMUNG IN IgG- UND IgM-FRAKTIONEN DES SERUMS UND VERGLEICH DER ERGEBNISSE MIT DEM NEUTRALISATIONSTEST*

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Zusammenfassung. Die Antikörperaktivitäten der IgM- und IgG-Fractionen des Serums sind mit der $\text{Al}(\text{OH})_3$ -gelchromatographischen Methode und dem Neutralisationstest stets im gleichen Verhältnis zu erfassen. Damit konnte nachgewiesen werden, daß beide Methoden sowohl bei der Überwachung von Immunisierungsaktionen, wie auch bei der Untersuchung von Patientenserum eingesetzt werden können.

Darüber hinaus führten diese Arbeiten zu einem einfachen Verfahren, an Sephadex getrennte Serumfraktionen weiter zu reinigen und Fraktionen, die IgM und IgG enthalten, weitgehend von Fremdproteinen zu befreien.

Der bisher gebräuchliche Nachweis von Antikörpern gegen Enteroviren ist der Neutralisationstest in Gewebekulturen. Unter Verwendung von $\text{Al}(\text{OH})_3$ -Gel wurde ein einfacher und schneller Nachweis von Antikörpern zuerst von THOMSEN [1] für Poliovirus Typ 1 ausgearbeitet. Er ging bei diesen chromatographischen Verfahren davon aus, daß die Polioviren unter genau definierten Bedingungen durch $\text{Al}(\text{OH})_3$ -Gel adsorbiert werden und sich mit einem geeigneten Elutionsmittel eluieren lassen. Die unterschiedliche Oberflächenbeschaffenheit der verschiedenen Poliovirusstämme bedingt eine verschieden feste Bindung der Viren an das Adsorbens und daraus folgend auch unterschiedlich hohe Konzentrationen an Phosphat-Ionen für die Elution. Das gleiche Adsorbens eignet sich auch für die chromatographische Trennung von Polioviren und der Poliovirus-Antikörperkomplexe, da Virus-Antikörperkomplexe anders an $\text{Al}(\text{OH})_3$ -Gel gebunden werden als das freie Virus. Die Anwendbarkeit dieser Methode in der Diagnostik setzt jedoch voraus, daß sich der chromatographische Antikörper-Nachweis bei Verwendung geeigneter Virusstämme auch mit den Typen 2 und 3 durchführen läßt [2].

Es wurden deshalb Virusstämme der Typen 2 und 3 auf ihre Eignung für die chromatographische Methode geprüft, d. h. darauf, ob ihre Antikörperkomplexe wie beim Typ 1, Stamm Mahoney, fester als das freie Virus an das Adsorbens gebunden werden. Je ein Stamm des Typs 2 (518/65) wie auch des Typs 3 (WM3) erwies sich als geeignet. Die oben erwähnte höhere Bindungsfestigkeit der Virus-Antikörperkomplexe gegenüber dem freien Virus drückt

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sich experimentell in einer höheren Phosphatpuffermolarität aus, die zur Elution dieser Komplexe erforderlich ist. Die Puffermolarität steht in eindeutiger Beziehung zur gebundenen Antikörpermenge und ist ein Maß für die Konzentration der vorliegenden homologen Antikörper. Voraussetzung für die Durchführung solcher Antikörperbestimmungen ist die Verwendung von ^{32}P -markierten Viren. Als Maß für die Viruskonzentration der Proben dient die Aktivität des im Virus eingebauten radioaktiven Phosphors.

Die chromatographische Bestimmung der Antikörper kann unter verschiedenen experimentellen Bedingungen durchgeführt werden [1, 2].

Es wurden die Antikörper gegen alle drei Poliovirustypen in einer großen Anzahl Serumproben chromatographisch bestimmt, die erhaltenen Titer denen des Neutralisationstestes gegenübergestellt und mathematisch-statistisch ausgewertet [3].

Es konnte festgestellt werden, daß zwischen den Ergebnissen beider Methoden keine signifikanten Unterschiede bestehen, obwohl bei den Typen 2 und 3 in beiden Methoden unterschiedliche Virusstämme als Antigene verwendet wurden.

Der Einsatz der chromatographischen Methode sowie die Überprüfung der erhaltenen Titerwerte erfolgten ausschließlich bei Seren von Gesunden, deren Antikörperverteilung auf die Haupt-Immunglobulinfraktionen des Serums (IgG und IgM) vermutlich eine andere ist als die im Serum von akut Erkrankten. Daraus ergibt sich die Frage nach der Erfäßbarkeit der in den IgM- und IgG-Fractionen des Serums vorhandenen Antikörper-Aktivitäten mit der $\text{Al}(\text{OH})_3$ -Gelchromatographie.

Um diese Frage zu klären, mußten zunächst Antikörperbestimmungen in isolierten IgM- und IgG-Fractionen des Serums vorgenommen werden, d. h. es mußte geprüft werden, wie sich die Immunglobulinfraktionen allein und im Komplex mit dem Virus des gleichen Typs an $\text{Al}(\text{OH})_3$ -Gel verhalten.

Als Modell wurden Kaninchen-Immunsereen gegen Stamm Mahoney und LSc2ab, die durch Schnellimmunisierung gewonnen wurden, eingesetzt. Alle Untersuchungen wurden mit ^{32}P -markiertem Poliovirus Typ 1, Stamm Mahoney durchgeführt.

Material und Methoden

Poliovirusstämme. Typ 1: ^{32}P -Mahoney [4].

Immunsereen. Kaninchen-Immunsereen gegen Polio-Typ 1, Mahoney und LSc2ab (Sabin).

Fraktionierung der Immunsereen an Sephadex G 200. Auf eine Sephadex-Säule (380 mm hoch, 33 mm Durchmesser) wurden 5 ml Immunsereum aufgetragen und mit einem Tris-Puffer (6,05 g Tris, 23,4 g NaCl, 1,0 g Na-azid und 27,5 ml $N\text{HCl}$ auf 1000 ml) eluiert. Die Fraktionen wurden mit einem Fraktionssammler zu je 3,3 ml aufgefangen und ihre Extinktionen bei 280 nm automatisch gemessen. (Gerät: LKB 4900 A-2 Atomic Re Cy Chrom System.) Die Fraktionen 16 und 17 enthielten den IgM-Anteil, die Fraktionen 27, 28, 29 den IgG-Anteil des Serums.

Fraktionierung der IgM- und IgG-Serumfraktionen an $Al(OH)_3$ -Gel. Verwendet wurde $Al(OH)_3$ -Gel C γ (Behring). In ein Zentrifugenröhrchen wurden nacheinander 1 ml 0,25% $Al(OH)_3$ -Gel und 2 ml der entsprechenden 1 : 5 verdünnten Immunglobulinfraktionen gegeben. Nach 20 min Adsorption bei Zimmertemperatur wurde kurz zentrifugiert und der Überstand vom $Al(OH)_3$ -Gel-Niederschlag scharf abgesaugt. Zur Elution der Immunglobuline dienten Phosphatpuffer steigender Molarität (0,005 bis 0,32 M), pH. 7,5. (Die Phosphatpuffer der Molarität 0,005, 0,0075, 0,01, 0,015, 0,02, 0,025, 0,03, 0,035, 0,04, 0,05, 0,06, 0,07, 0,08, 0,10, 0,12 und 0,14 wurden mit 0,05 Tris-Puffer pH 7,5 hergestellt, die Puffer der Molarität 0,16, 0,18, 0,22, 0,24 und 0,32 mit Aqua dest.) Nach 20 min Elution bei Zimmertemperatur wurde abermals zentrifugiert und mit allen Puffern nacheinander eluiert.

Die Extinktionen aller Eluate wurden nun bei 260 nm spektrophotometrisch gemessen und damit ihr Proteingehalt bestimmt.

Chromatographischer Antikörpernachweis. Die Verdünnung der Immunseren sowie der IgM- und IgG-Fraktionen erfolgte in log 2-Stufen (1 : 250 bis 1 : 64 000) mit einem Gemisch aus Hanks-Lösung und 0,025 M Tris-Puffer im Verhältnis 1 : 1, pH. 7,5. Von diesen Verdünnungen wurden je 0,5 ml in ein Zentrifugenröhrchen pipettiert. Außerdem wurde ein Röhrchen ohne Serum, nur mit Hanks/Tris-Gemisch als Viruskontrolle mitgeführt. Zu den Serumverdünnungen und dem Kontrollröhrchen wurden 0,5 ml Virusverdünnung (in einer Konzentration von etwa 5000 Imp/min/ml) gegeben und die Serum-Virusgemische 3 Std. bei 37 °C im Wasserbad und anschließend über Nacht bei 4 °C inkubiert. Danach wurde mit 1 M Acetatpuffer pH 5,5 eine pH-Korrektur auf etwa pH 6,0 vorgenommen und 0,5 ml 0,25%iges $Al(OH)_3$ -Gel ausgegeben. Nach kräftigem Schütteln blieb das Gemisch 20 min bei Zimmertemperatur zur Adsorption stehen. Anschließend wurde zentrifugiert und der Überstand scharf abgesaugt.

Zur Elution von nicht mit Antikörpern beladenem Virus wurde ein Phosphatpuffer benutzt, dessen Molarität etwa in der Höhe der EC_{50} -V des freien Virus lag (für Mahoney 0,01 M).

Nach Aufschütteln des Sediments blieb das Gemisch 20 min bei Zimmertemperatur zur Elution stehen und wurde anschließend wiederum zentrifugiert. In den Überständen wurde die Viruskonzentration bestimmt (Imp/min/ml). Die Viruskonzentration des Kontrollröhrchens wurde gleich 100% gesetzt (E_{max}) und die in den unteren Eluaten erhaltenen Aktivitäten als Prozente von E_{max} ausgedrückt. Aus den so ermittelten und graphisch dargestellten Werten konnte die Serumverdünnung abgelesen werden, bei der 50% des nicht mit Antikörpern beladenen Virus eluiert werden (Abb. 1 und 2). Die so erhaltenen Verdünnungen wurden als Geladsorptionstiter (GAT) definiert.

Neutralisationstest. Die inaktivierten Immunseren und die gegen Hanks-Lösung dialysierten IgM- und IgG-Fraktionen wurden mit Nährmedium in log 2-Stufen verdünnt und gegen 100 TCD₅₀ der Poliovirusstämme Mahoney und LSc2ab angesetzt. Nach 3stündiger Inkubation bei 37 °C und Aufbewahrung über Nacht bei 4 °C wurden 0,2 ml dieses Virus-Serum-Gemisches in Affennieren-Zellkultur Röhrchen gegeben und nach 30 min Adsorption mit 1 ml Nährmedium aufgefüllt. Die mikroskopische Ablesung wurde nach 5tägiger Bebrütung bei 37 °C vorgenommen.

Ergebnisse

Aus den Abb. 1 und 2 ist klar ersichtlich, daß ein Vergleich der Geladsorptionstiter mit den Titerwerten des Neutralisationstestes sowohl für die Immunseren gegen Mahoney und LSc2ab wie auch für die isolierten IgM- und IgG-Fraktionen eine gute Korrelation zeigt. Außerdem wird auf diesen Darstellungen deutlich sichtbar, daß die chromatographische Antikörperbestimmung etwa um den Faktor 10^2 empfindlicher ist als der Neutralisationstest.

Damit ist eindeutig erwiesen, daß mit der chromatographischen Methode Antikörper in beiden Immunglobulinfraktionen nachgewiesen werden können und daß sie außerdem im gleichen Verhältnis erfaßt werden wie im Neutralisationstest.

Die Fraktionierung der durch Sephadex-Filtration erhaltenen IgM- und

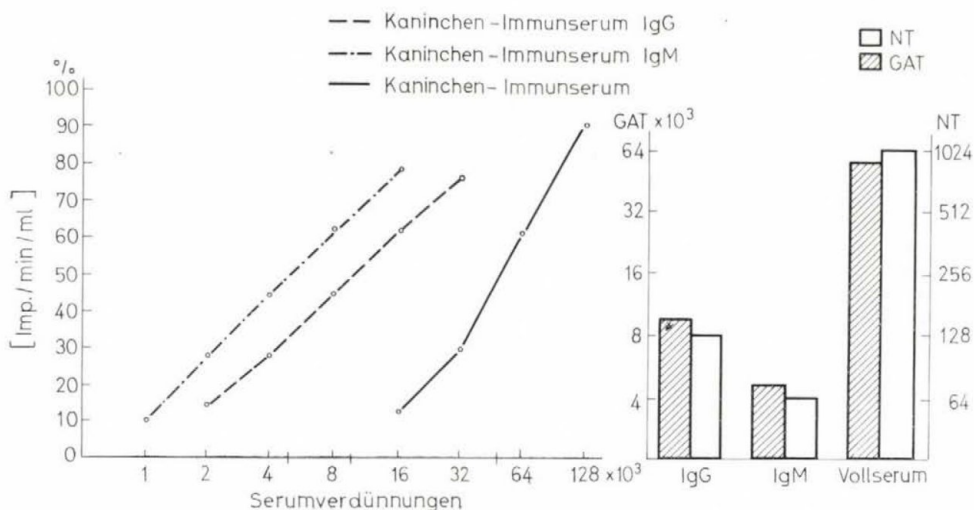


Abb. 1. Bestimmung von Polio-Antikörpern mit $\text{Al}(\text{OH})_3$ -Geladsorption und Vergleich der Ergebnisse mit dem Neutralisationstest Polio-Typ 1 Mahoney

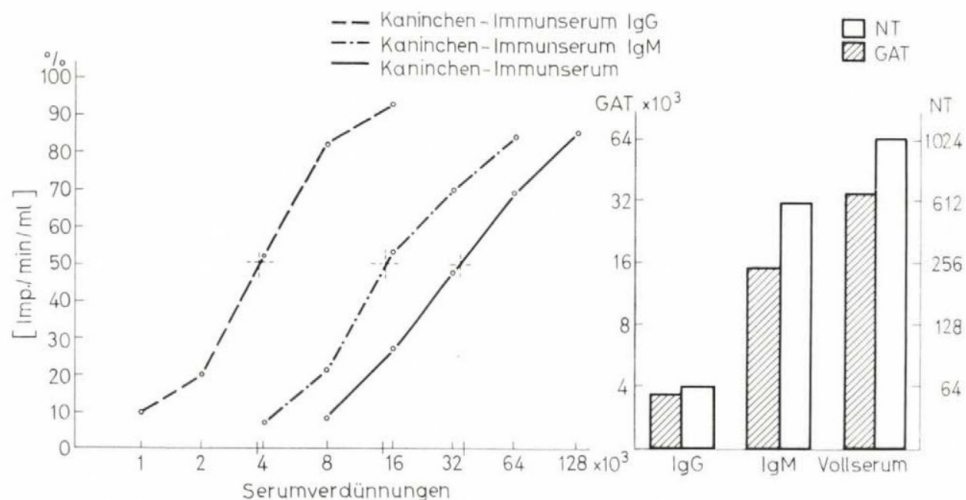


Abb. 2. Bestimmung von Polio-Antikörpern mit $\text{Al}(\text{OH})_3$ -Geladsorption und Vergleich der Ergebnisse mit dem Neutralisationstest Polio-Typ 1 LSc2ab

IgG-Anteile des Serums an $\text{Al}(\text{OH})_3$ -Gel gab Aufschluß über die Reinheit der Präparate.

Wie aus der Abb. 3 ersichtlich ist, erwies sich die an Sephadex gereinigte IgG-Fraktion als nicht einheitlich. Mehrere kleinere Protein-Peaks treten neben einem Elutionsmaximum auf, das bei etwa 0,015 M Phosphatpuffer liegt.

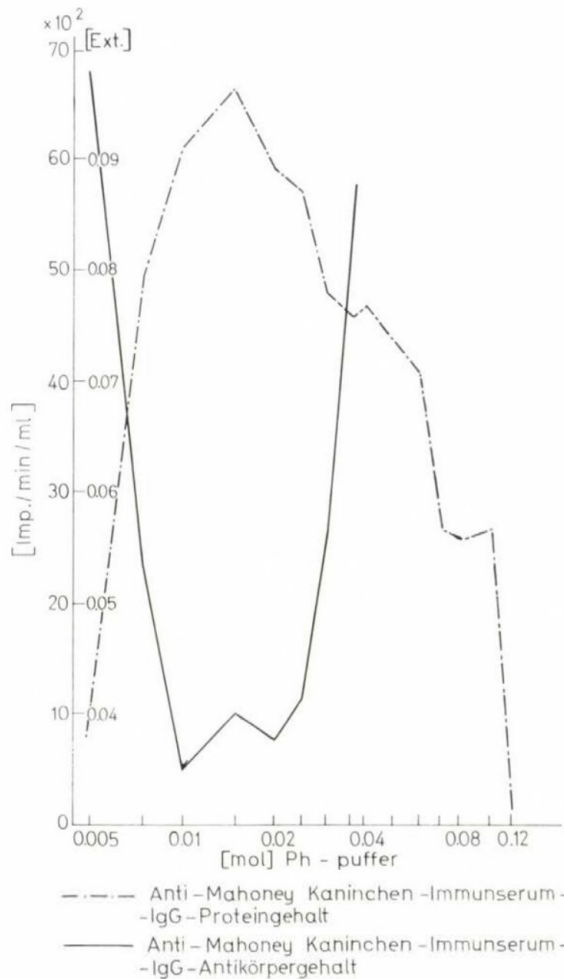


Abb. 3. Auftrennung von Polio-(Typ 1)-IgG-Fractionen an $Al(OH)_3$ -Gel und deren Anti-körpernachweis

Das gleiche gilt auch für die Serumfraktion, die IgM enthält. Hier liegt das Elutionsmaximum zwischen 0,04—0,08 M Phosphatpuffer.

Diese Ergebnisse zeigen, daß auch an $Al(OH)_3$ -Gel bei der Elution von Serumfraktionen eindeutige Unterschiede im Verhalten der IgM- und IgG-Anteile vorliegen. Der nächste Schritt war der Nachweis der Antikörperaktivitäten, um die Frage zu klären, ob in allen Protein-Peaks Antikörperaktivität gegen Polio Typ 1 vorlag. Dieser Nachweis wurde mit der Gelchromatographie und ^{32}P -Mahoney-Viren durchgeführt. Die Abbildungen 3 und 4 zeigen eindrucksvoll, daß nur die beiden Protein-Elutionsmaxima Antikörperaktivität aufweisen, die sich in einer gegenläufigen Kurve darstellen.

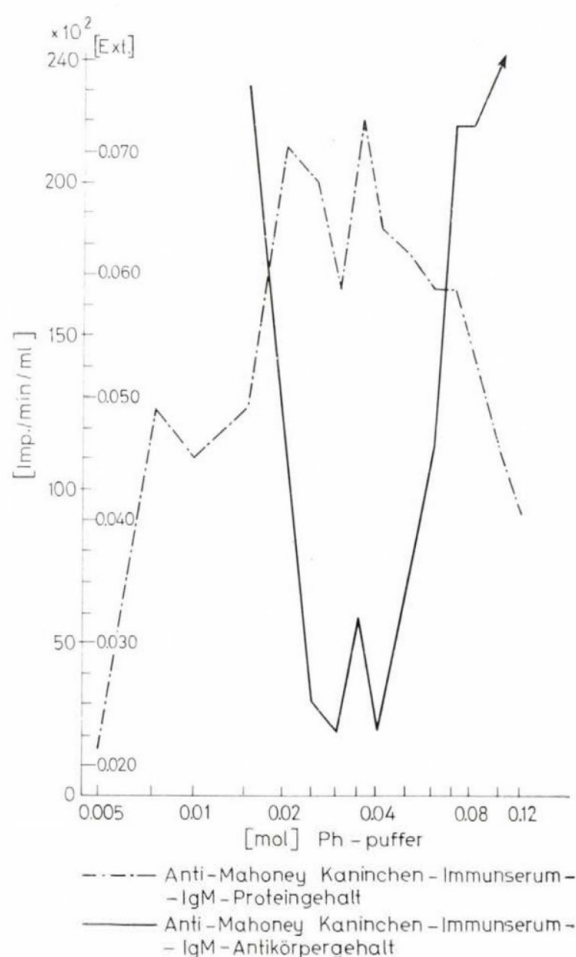


Abb. 4. Auftrennung von Polio-(Typ 1)-IgM-Fraktionen an $\text{Al}(\text{OH})_3$ -Gel und deren Antikörpernachweis

In gleicher Weise wie die bereits an Sephadex gereinigten Serumfraktionen wurde auch Vollserum kontinuierlich von $\text{Al}(\text{OH})_3$ -Gel eluiert und in den erhaltenen Eluaten die Antikörperaktivität chromatographisch bestimmt.

Wie die Abb. 5 zeigt, treten auch beim Vollserum deutliche Unterschiede zwischen der Eluierbarkeit der IgM- und IgG-Anteile auf. Der Nachweis der Antikörperaktivität gelingt daher im Vollserum mit den gleichen Puffermolaritäten wie bei den isolierten IgM- und IgG-Fraktionen.

Wenn sich der Nachweis von Antikörperaktivitäten in den IgM- und IgG-Anteilen des Vollserums führen läßt, bietet sich mit dieser Methodik ein Weg an, eine zusätzliche Aussage bei der Klärung der Ätiologie von Virus-erkrankungen zu erhalten.

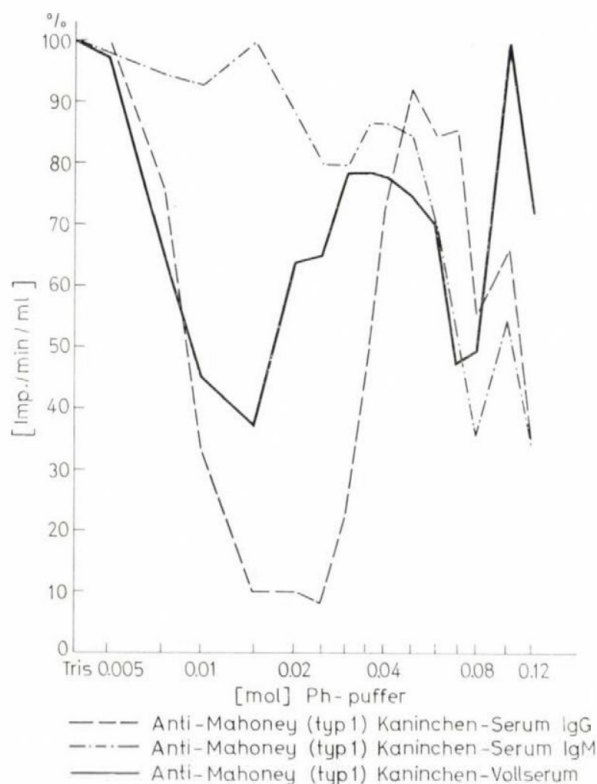


Abb. 5. Antikörpernachweis in der IgG- und IgM-Fraktion des Vollserums

Eine Einsatzmöglichkeit der von Fremdproteinen weitgehend befreiten spezifischen Antikörper enthaltenden Serumfraktionen wäre u.a. bei der Immunofluoreszenz-Technik denkbar.

Die Verfasser danken Fr. Dr. ESZTER KUKÁN für die Durchführung der Neutralisationsteste und Fr. VERA KUSSAT sowie Fr. ANNELIESE HEROLD für ihre wertvolle Mitarbeit bei der Durchführung der Versuche.

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DEVELOPMENT OF VACCINATION IMMUNITY IN HOSPITAL PERSONNEL REVACCINATED AT THREE-YEAR INTERVALS

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Summary. The immunity induced in hospital personnel by two consecutive successful revaccinations carried out at an interval of three years has been studied. The vaccinees had been primovaccinated in infancy and revaccinated at school age. The antibody responses and the persistence of the antibodies were investigated.

Three years after the last revaccination, all vaccinees had neutralizing antibody levels not lower than primovaccinated children three weeks after vaccination. The neutralizing-antibody response to the last vaccination was less intensive than that given to the previous one. The haemagglutination-inhibiting antibody response also decreased with the growing number of previous vaccinations, suggesting that the resistance to vaccinia virus was increasing.

It is concluded that repeated revaccinations lead to a vaccination immunity ever increasing in level and duration. Consequently, the revaccination interval of health personnel at high risk may be prolonged after two successful triennial revaccinations.

A single vaccination affords protection from smallpox for 4 to 5 years [1, 2]. Although the duration of immunity after revaccination has not been studied by epidemiological methods, serological investigations have shown that neutralizing (NE) antibodies appear in higher concentrations and persist longer in sera of revaccinees than in those of primovaccinees [3]. In many countries, personnel likely to be exposed to smallpox are vaccinated every 3 years. Such triennial revaccination is mandatory for health workers at risk in Hungary as are vaccination in infancy and revaccination at school age.

These requirements have permitted a long-term study of local and antibody responses following repeated vaccinations [4, 5]. Recent studies have supplied further data on the immunity of the same subjects, and primovaccinated children have been examined similarly.

In the present work, observations of the development of the immunity resulting from triennial revaccination and the efficacy of this revaccination system are discussed.

Materials and methods

Vaccination history of subjects studied. *Group A.* One-year-old unvaccinated children. *Group B.* Four-year-old children who had successfully been primovaccinated at 1 year of age. *Group C.* Men and women aged 20 to 60 working in a hospital for infectious diseases. They had successfully been primovaccinated in infancy and revaccinated at school age.

In Groups B and C, characteristic vaccination scars were regarded as proof of successful primovaccination, but successful revaccination could not be established with certainty.

Vaccine. The vaccine (Human, Budapest) was glycerolated bovine lymph. For the third vaccination of Group C performed in 1963, a vaccine with 7.10^6 PFU/ml was used. All the other vaccinations were carried out with vaccines having a potency over 10^8 PFU/ml.

Vaccination and sampling. Primary vaccination was performed at two distant sites on the upper arm, using a single linear scratch 1 to 2 mm in length for each. Revaccination was more vigorous, doubly-crossed linear scratches 5 to 7 mm in length were made at two sites. Local reactions were read on the 7th day after vaccination and regarded as successful only if at least one "major reaction" consisting of a pustule or scab surrounded by an area of palpable induration had occurred.

Members of Group A were primovaccinated, and bled 3 weeks after successful vaccination. These samples were considered characteristic of the antibody response to the first smallpox vaccination.

Members of Group B were not vaccinated, but bled to provide information on the antibody status 3 years after successful primovaccination.

Members of Group C were vaccinated twice with an interval of 3 years. Since they had been vaccinated twice in childhood, these triennial vaccinations represented their third and fourth smallpox vaccinations. Blood was taken before and 3 weeks after successful vaccination for demonstrating the antibody response to the third and fourth vaccinations. The samples drawn before the last (fourth) vaccination provided information also on the antibody status 3 years after the third vaccination.

An additional sample was drawn 3 years after the last vaccination in order to investigate the 3-year antibody status after the fourth vaccination.

Antibody assay. NE antibody was titrated in the serum samples by pock inhibition on the chick chorio-allantois [4]. The first dilution was 1:4 uniformly. A 60% reduction was regarded as end-point. Fourfold dilution series were prepared except from the Group C serum samples taken before, and 3 weeks after, their third vaccination. For the sake of comparison, the titres of these sera were corrected by reducing titres 1:8, 1:32 and 1:128 to 1:4, 1:16 and 1:64, respectively.

Haemagglutination inhibition (HAI) titrations were performed with the Takátsy Micro-titrator.

A reference serum was included with each batch of titrations so that results remained comparable although the investigation lasted several years.

The assay data were analysed statistically. The comparison of titres was based on the $-\log_2$ transformation of titre values. The geometric means were compared using Student's *t* test. Classifying data were evaluated by the χ^2 test, choosing $P = 0.05$ as fiducial limit.

Results

1. Persistence of anti-vaccinia antibodies

Table I shows the immune status of the vaccinees three years after successful vaccination for the 1st, 3rd and 4th time.

Table I

*Serological status of subjects with different vaccination history,
3 years after the last vaccination*

Group	No. of previous vaccinations	No. of vaccinees	Per cent of seropositives	Geometric mean of titres of seropositives
NE antibodies				
B	1	13	69.2	5.7
C	3	45	89.9	6.9
C	4	19	100.0	13.9
HAI antibodies				
B	1	13	61.5	11.3
C	3	47	60.0	7.5
C	4	19	42.2	6.5

As regards the persistence of NE antibodies it can be concluded from the results that

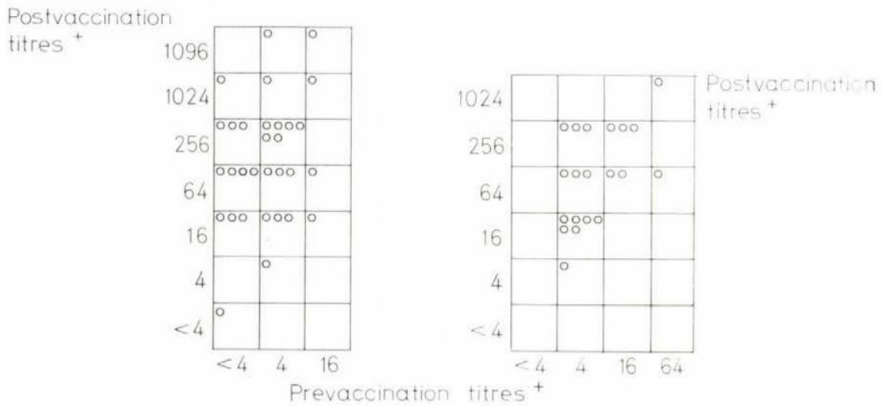
(a) the frequency of seronegative subjects decreased with the increasing number of previous vaccinations;

(b) the geometric mean for the subjects vaccinated four times remained significantly higher than that for the primary vaccinees ($t_{26} = 2.547, P < 0.02$).

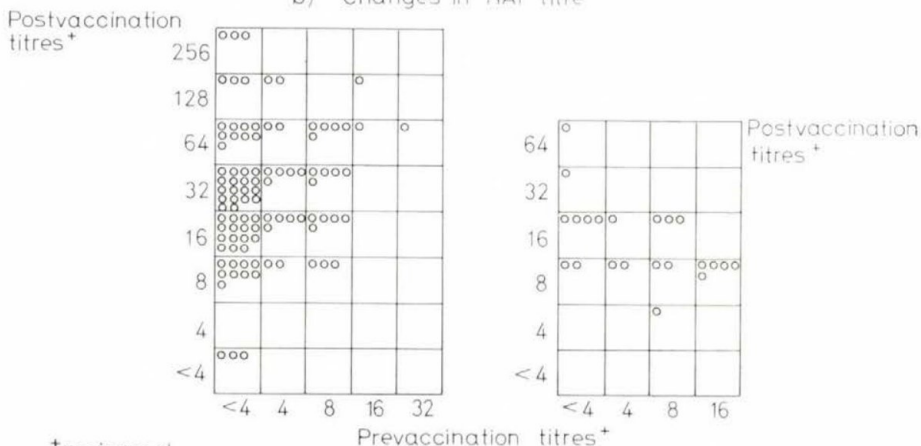
Antibody response to successful revaccination

Group	C	C
No of previous vaccinations	2	3

a) Changes in NE titre



b) Changes in HAI titre



*reciprocal

Fig. 1

The antibodies measured by HAI were different in behaviour from those measured by virus neutralization; neither the geometric mean nor the frequency of seronegative vaccinees was significantly different in subjects with a different vaccination history.

2. Antibody response to successful vaccination

The postvaccination antibody responses are shown in Fig. 1 and Table II. The following conclusions can be drawn.

Table II

Antibody response to successful vaccination of subjects with different vaccination history

Antibody	Group	No. of previous vaccinations	No. of vaccinees tested	Reactors, per cent	G. M. for titres of	
					prevaccination sera	postvaccination sera of reactors
NE	A	0	27	92.6	—	9.2
	C	2	31	90.3	3.6	137.1
	C	3	20	90.0	7.2	73.6
HAI	A	0	29	100.0	—	29.8
	C	2	97	85.6	2.3	32.0
	C	3	22	63.6	4.8	19.7

NE antibody response occurred in about 90% of the vaccinees, and the frequency of reactors appeared to be unrelated to the vaccination history. The postvaccination titres, on the other hand, were higher after revaccination than after primary vaccination.

The frequency of HAI reactors decreased as the number of previous vaccinations increased. Primary vaccination induced HAI seroconversion in each vaccinee, whereas the 3rd and 4th vaccinations were followed by an at least 4-fold titre rise in 87 of 97 and 14 of 22 vaccinees, respectively. The difference is significant ($\chi^2 = 12.935$; $P < 0.001$). The postvaccination HAI titres of the revaccinees were not higher than those of the primovaccinees. The individual titre changes accompanying revaccination are shown in Fig. 1. Both the NE and HAI titres changed less after the 4th than after the 3rd vaccination. The geometric mean for the titre rises following the 3rd vaccination exceeded that following the 4th vaccination (in relation of the changes in the NE and HAI titres, $t_{44} = 3.067$; $P < 0.01$; and $t_{95} = 2.22$, $P < 0.05$, respectively).

Discussion

In estimating the degree of protection of vaccinees we relied on their NE and HAI antibody titres. As regards the protection from smallpox, the significance of the two kinds of antibody is different. The anti-infectious capacity of the serum is characterized by its NE antibody titre which, consequently, may

be regarded as the best indicator of the donor's immunity [2]. The rate of HAI antibody production, on the other hand, depends on the intensity of virus multiplication in the organism and HAI antibodies have no protective activity [7]. Accordingly, the serum HAI antibody content offers no information on the actual level of immunity, but the intensity of the HAI antibody response to revaccination may be regarded as an indicator of resistance to vaccinia virus.

The present results show a well-defined difference between primovaccinees and revaccinated subjects in the level of protection. Three years after primary vaccination, there was no NE antibody in the serum of 31% of the vaccinees, whereas no sample was found seronegative three years after the 4th vaccination. Furthermore, a comparison of the data in Table I with those in Table II shows that the NE titres measured 3 years after the 4th vaccination were practically equal to those measured three weeks after primary vaccination.

The depressed NE antibody response to the 4th vaccination agrees well with the general experience that the antibody response to reinfection is suppressed by pre-existing protective antibody.

The presence of HAI antibodies was related to an increased resistance to vaccinia virus. Of the subjects vaccinated for the 4th time 64%, of those vaccinated for the 1st time 100%, gave an HAI antibody response. Besides, the HAI antibody response to the 4th vaccination remained below that to the 3rd vaccination.

The above data suggest that the protection is not only renewed but also corroborated by successive revaccinations.

Reflecting these observations to the triennial revaccination system, we can state that two successful revaccinations at an interval of three years result in a very favourable immune status in adults who had successfully been vaccinated in infancy. The protection of such subjects is characterized by the fact that their serum antibody titres found in sera at the due time of the next revaccination (according to the triennial system) are not lower than the titres three weeks after successful primary vaccination. Such antibody responses suggest that as the number of revaccinations increases, the intervals might well be prolonged and the time of the next vaccination of hospital personnel should be determined by serological monitoring. Such a prolongation of the vaccination interval seems to be justified by the reduced antibody response that followed the 4th vaccination.

It may be stressed that all our results are related to successful vaccination (take). Consequently, our proposal concerning the prolongation of the interval between successive revaccinations is only valid if the results of consecutive vaccinations are regularly checked.

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DEFECTIVE LYSOGENY IN BACILLUS ANTHRACIS STERNE STRAINS

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Summary. Strains CN 18–74 and CN 35–18 of M. STERNE's collection, characterized as very stable acapsulogenic mutants suitable for immunizing domestic animals, were shown to be defective lysogenic strains. Induction with mitomycin C resulted after a slight residual growth in lysis. Electron microscopy detected phage structures in the purified lysates. Tests on a number of indicator strains failed to show infective phage particles. The two strains differed in the morphology of defective phages. From strain CN 18–74 a derivative not sensitive to mitomycin C induction was isolated.

ALTENBERN and STULL [1] have shown that several strains in the genus *Bacillus* are, after a slight residual growth, lysed as an effect of inducing agents such as mitomycin C. According to their findings, during the lysis of *Bacillus cereus* strain 6464 a toxin accumulates in the culture. They showed a similar behaviour of other *B. cereus* strains and of *B. subtilis*, *B. brevis*, *B. vulgatus*, *B. thuringiensis* and *B. anthracis*. With most of the strains they did not examine the cause of lysis after mitomycin C induction. As *B. anthracis* strains isolated by STERNE [2] are widely used for the immunization of animals, [3] it seemed interesting to investigate the problem.

Materials and methods

Culture medium. YP (yeast—peptone) broth was used [4]. Solid medium was prepared by adding 1.5% agar to YP broth.

Bacterial strains. Apathogenic and acapsulogenic *B. anthracis* strains CN 18–74 and CN 35–18 of M. STERNE's collection were used. A derivative of strain CN 18–74 not lysed after mitomycin C induction was obtained in our laboratory and designated as CN 18–74/cur. Indicator strains for showing the presence of infective phage particles were capsulogenic cultures 81C+, 88C+ and 92C+ and acapsulogenic cultures Davis, VC–, 69C–, 77C– and 99C– of *B. anthracis* of our collection. The presence of bacteriocins was attempted to prove with strains *B. megaterium* Mutilans C and *B. cereus* 4. Dilution of the lysates was performed with a solution containing 1 part YP medium and 4 parts saline.

Induction of lysis. The strain was cultured overnight, then inoculated into fresh YP medium and shaken in water bath at 37°C. The optical density of growth was measured by the use of a Bausch-Lomb spectrophotometer set at 620 mμ wavelength. The optical densities and the corresponding cell counts (calculated on the basis of colony-forming units corrected with the average chain length) are shown in Fig. 1. At O.D. 0.2 mitomycin C was added at different concentrations and the cultures were reincubated and measured spectrophotometrically.

Concentration and purification of lysates for electron microscopy. Cultures of exponential phase growth were transferred into 1–3 litres of fresh YP broth; the inoculum had been prepared by shaking 100 ml cultures in 1 litre Erlenmeyer flasks and inducing with 0.5 μg mito-

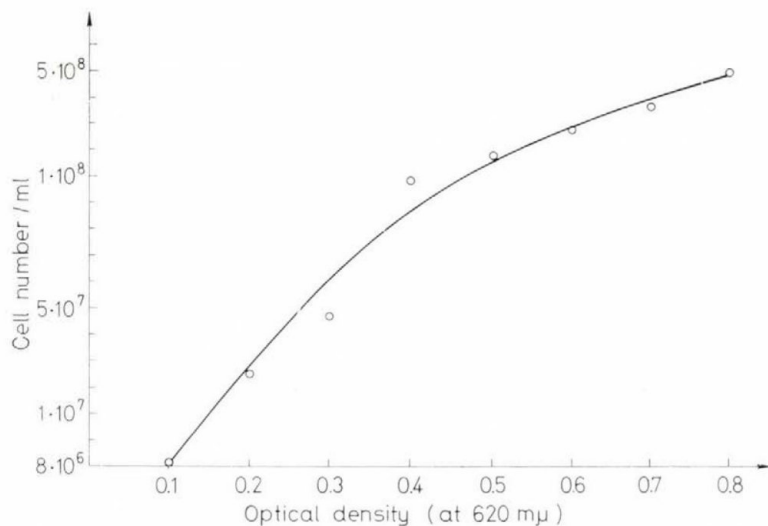


Fig. 1. Cell numbers of *B. anthracis* Sterne strains at different optical densities of the cultures. Corrections were made by considering the number of colony-forming units and average chain length

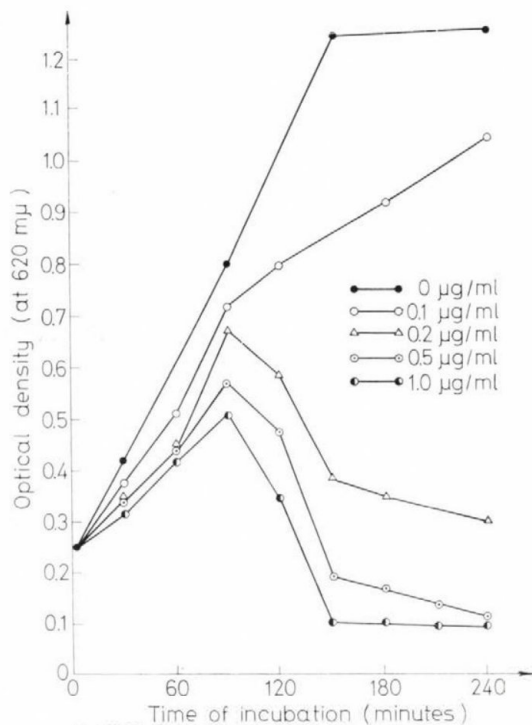


Fig. 2. Lysis induction of *B. anthracis* strain CN 35-18 with mitomycin C at different concentrations, added at zero minute

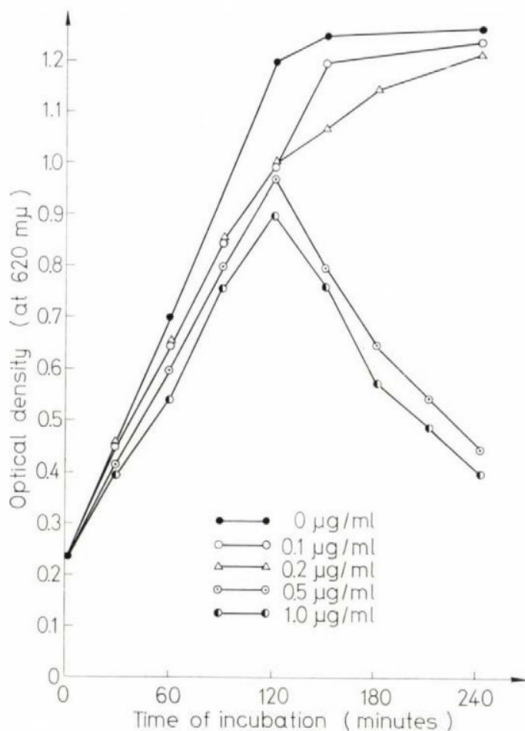


Fig. 3. Lysis induction of *B. anthracis* strain CN 18-74 with mitomycin C at different concentrations added at zero minute

mycin C per ml. At maximum lysis, or 4 hours after induction of strain CN 18-74/*cur*, the cultures were united and the bacterial debris was removed by centrifugation at 5000 *g*. The lysate obtained in this manner was concentrated by the method of YAMAMOTO *et al.* [5] slightly modified as follows. After dissolving 5% polyethylene glycol (PEG) 6000 and 0.5 *M* NaCl, the lysate was adjusted with *N* HCl to pH 6.0 and stirred in the refrigerator for 1 hour. After the appearance of opalescence, the lysate was left to stand in the refrigerator for 4 hours, then centrifuged at 5000 *g* for 45 minutes. The deposit was resuspended in 1/100 of the original volume of the lysate in a buffer consisting of 0.05 *M* Tris HCl pH 7.5; 0.01 *M* magnesium acetate; 0.11 *M* ammonium acetate and 0.001 *M* mercaptoethanol. Cyclic centrifugation was then performed in the Janetzki VAC 6 ultracentrifuge at 30 000 *g* for 2 hours, then at 8000 *g* several times for 10 minutes. The homogeneous opalescent liquid was mounted on carbon-impregnated Formvar film placed on LKB grid; the excess liquid after 60 seconds adsorption was removed with filter paper. The preparation after washing with distilled water three times was stained with uranyl acetate [6]. The examinations were made with a Jem-100 B type electron microscope.

Attempts at showing infective phage particles and bacteriocin. Cultures showing maximum lysis after the addition of mitomycin C were filtered through collodion membrane and various dilutions of the filtrate were spotted on soft agar layer seeded with the indicator strains and poured over YP plates [7].

Results

Induction of lysis. Growth curves for *B. anthracis* strains Sterne CN 18-74 and CN 35-18 after induction with various concentrations of mitomycin C are shown in Figs 2 and 3. The two strains differed somewhat in

behaviour. Strain CN 35—18 showed a definite and fairly rapid lysis after the addition of 0.2, 0.5 or 1 μg mitomycin per ml. The lysis of strain CN 18—74 at 0.2 μg mitomycin concentration was weak, but at 0.5 and 1 μg concentrations it was rapid although less marked than that of strain CN 35—18.

An attempt was made to isolate derivatives devoid of the lytic principle. The method was successful with strain CN 18—74 but failed with strain CN 35—18. The process of the so-called curing was as follows (Fig. 4). After lysis following the first induction with mitomycin a subculture was started with the

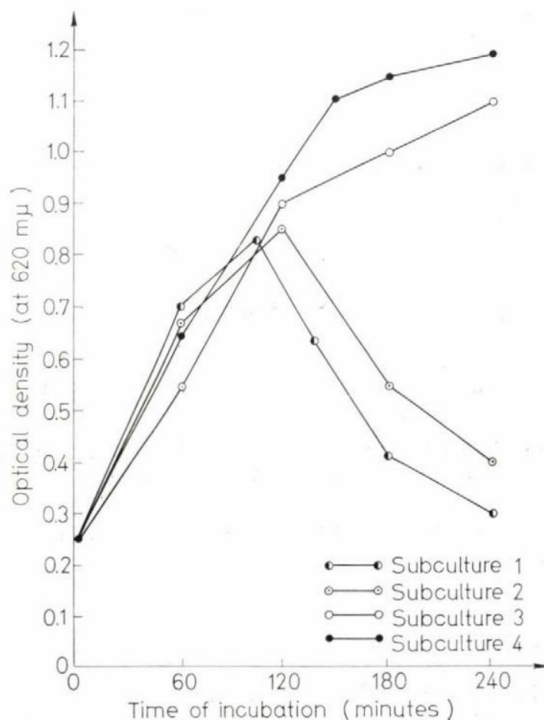
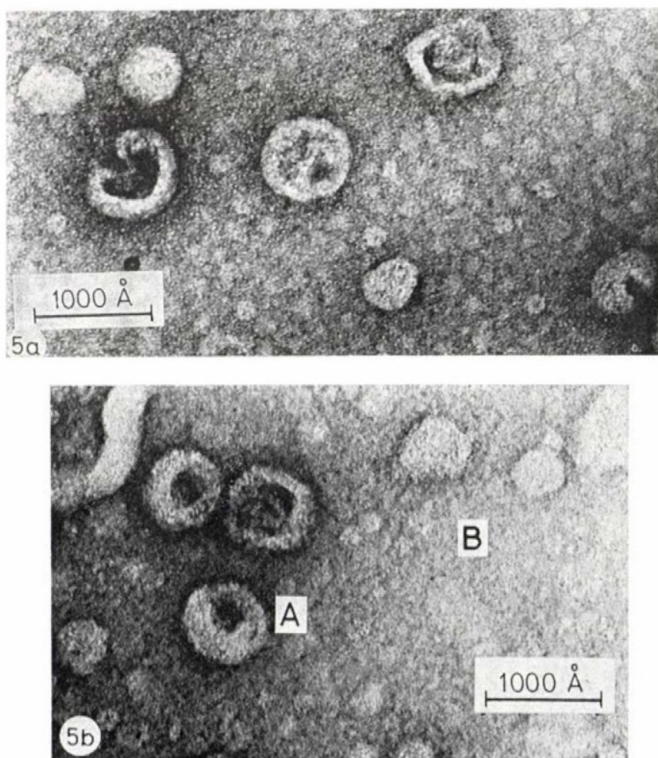


Fig. 4. Curing of strain CN 18—74 by serial induction with mitomycin C (0.5 $\mu\text{g}/\text{ml}$)

remaining intact bacteria. The same procedure was repeated several times. While the 2nd subculture differed only slightly from the 1st one, at the 3rd and 4th passages no lysis appeared. Curing was probably a result of a certain selection, since the degree of lysis decreased gradually with the number of subcultures. The subculture of strain CN 18—74 showing no lysis was grown with shaking until sporulation. The homogeneous spore suspension was streaked onto agar plates and 50 of the developing individual colonies were inoculated into separate tubes of broth and the absence of lysis was checked for each isolate. One of these isolates was designated as CN 18—74/*cur* and used in subsequent experiments. A similar treatment of strain CN 35—18 was un-

successful even after 8 subcultures, when after the addition of 0.5 μg mitomycin per ml, the degree of lysis was nearly identical with that shown by the parent culture.

Attempts at showing infective phage particles and bacteriocin. From lysates of the two strains the bacteria were removed by filtration, then the filtrate was spotted on indicator plates inoculated with 3 capsulogenic and 5 acapsulogenic *B. anthracis* strains. These experiments failed to demonstrate the presence of



Figs 5a and 5b. Electron micrographs of the concentrated lysate of *B. anthracis* strain CN 18-74. Stained with 2% uranyl acetate, $\times 150\,000$

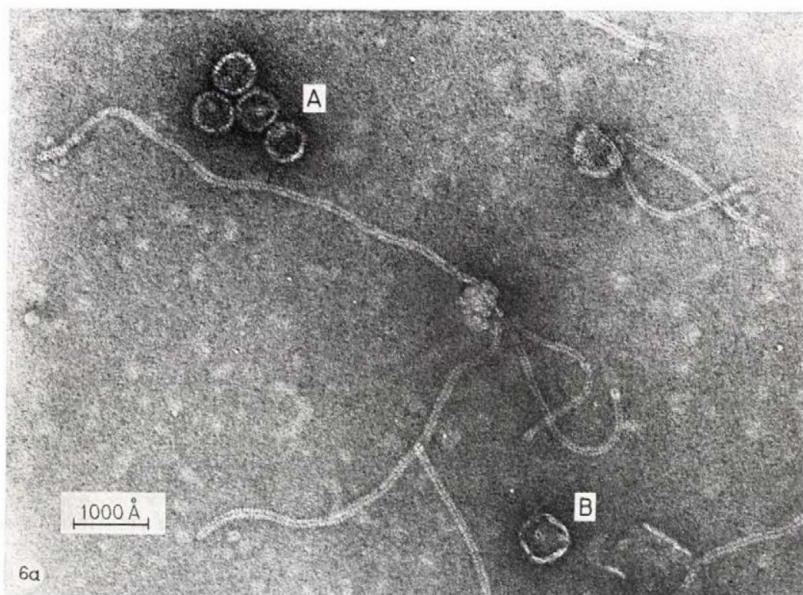
either infective phage particles or bacteriocins. Bacteriocins were not demonstrable with additional indicator strains (*B. megaterium* and *B. cereus*).

Electron microscopic studies. Various amounts of cultures were concentrated and purified by centrifugation: 3 litres for strains CN 18-74 and CN 18-74/*cur* and 1 litre for strain CN 35-18. In evaluating the pictures, the morphological characterization and nomenclature recommended by BRADLEY [8] were considered.

The electron microscopic picture of the lysate of strain CN 18-74 (Figs 5a and 5b) showed hollow structural elements nearly identical in size (A).

These corresponded probably to hollow phage heads with an average diameter of approximately 800 Å; the disrupted form seen had probably been damaged during ultracentrifugation. Homogeneous forms designated with B may have been defective virus particles or artificial products. The cured derivative of the same strain showed no similar formations. This finding was in agreement with the observation that the latter was not lysed after mitomycin treatment.

Electron micrographs obtained from strain CN 35-18 differed considerably from the above picture (Figs 6a and 6b). Hollow, mostly monodisperse forms



Figs 6a and 6b. Electron micrographs of the concentrated lysate of *B. anthracis* strain CN 35-18. Stained with 2% uranyl acetate, $\times 125\,000$

approximately 560 Å in diameter, seemingly corresponding to phage heads were seen (A); a few similar, but larger forms were also present (B). It is not clear whether forms A and B were identical in origin and differed in size only because of a deformation. In addition to these particles, several flexible forms (approximately 80 Å in width and varying in length) were seen. They showed a definite cross-striated structure and were hollow. The filamentous forms were, apart from their length, similar in morphology. There is no proof as to whether they corresponded to phage tails, since no connection with the heads could be demonstrated and they varied considerably in length.

TAKEYA *et al.* [9] have shown very similar forms in a purified pyocine 28 preparation. Pyocine 28 is supposed to be a defective phage tail. On the basis of this analogy we considered the particles in question to represent defective phage tail formations. The fact that the lysate of the corresponding strains failed to show a bacteriocin effect on indicator strains, does not disprove this conception. Intact phage particles could not be demonstrated in the preparations (the pictures shown represent several repeated electron microscopic examinations).

Discussion

STERNE's acapsulogenic *B. anthracis* strains CN 18—74 and CN 35—18 [2] have advantageously been applied for the vaccination of domestic animals [3, 10]. The strains are highly stable, that is, they do not revert into the capsular form [11]. The present examinations have shown that mitomycin C treatment induces lysis after a slight residual growth and the lysate contains electron microscopically demonstrable defective phage particles. The two strains differed partly in the morphology of the defective phage structures carried, partly in curability. The question arises, when had the cultures been infected with phages. The infection may have occurred during their isolation in CO₂ atmosphere by STERNE. This assumption has been supported by our experiments: under similar conditions we have isolated acapsulogenic and defective phage-carrier derivatives from strain Vollum after treatment with phage W [12]. In orientation electron microscopic studies these derivatives were not shown to contain defective phage particles, probably because UV irradiation and mitomycin C induced but a very slight degree of lysis in these cultures. The carrier-ship of defective phages was, however, proved by the fact that the organism showed a full immunity to the homologous phage and remained sensitive to heterologous phages [12]. Lysogenic *B. anthracis* strains are known to occur in nature [13]. We have attempted to isolate acapsulogenic variants by the CO₂ method from 4 lysogenic *B. anthracis* strains (HBA 30, 123, 125, 130) kindly supplied by Dr. EISENSTARK. Acapsulogenic strains obtained in this manner

carried infective and not defective phages. These experiments, accordingly, failed to demonstrate that natural lysogenic *B. anthracis* strains may split off defective lysogenic acapsulogenic cultures (unpublished data). The question arises as to whether the stable acapsulogenic property of strains CN 18-74 and CN 35-18 was associated with the lysogenic state. It may be assumed that in the presence of defective phages no $C^- \rightarrow C^+$ mutation occurs. The present experiments have shown a new characteristic, the defective lysogenicity, of the vaccine strains.

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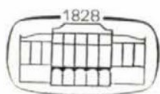
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REGULATED BIOSYNTHESIS OF PROTEASE AND ALPHA-AMYLASE IN *BACILLUS SUBTILIS*

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(Received February 14, 1972)

Summary. Two possibilities have been found for the regulation of alpha-amylase and protease production in *Bacillus subtilis* strain BS(IV)1968. In culture medium containing sodium citrate the biosynthesis of protease, in ammonium free medium that of alpha-amylase was inhibited. The extent of inhibition was not affected by the quality and quantity of the protein source.

It has long been known that fungi and bacteria produce alpha-amylase and protease enzymes. This industrially important question has been dealt with by a number of workers [1—10].

The optimum conditions for alpha-amylase production of *Bacillus subtilis* have been established by HAGIHARA [4]. His culture medium consisted of dibasic ammonium phosphate, sodium citrate, potassium chloride, soluble starch and microelements. COLEMAN and ELLIOT [11] were able to increase the yield of alpha-amylase by adding ferric ions to the culture medium. Their experiments showed that potassium biphosphate and dibasic sodium phosphate could be substituted for dibasic ammonium phosphate. No reference is, however, found in any of the above mentioned papers [1—11] to the correlation between protease and alpha-amylase production of *B. subtilis*, just as evidence is lacking concerning the regulation of the total yield and ratio of the two enzymes. Even among reports dealing with enzyme preparation only that of MUZI *et al.* [12] suggests that in the crystallization of alpha-amylase produced by *B. subtilis*, the disturbing effect of protease could be eliminated with diisopropyl fluorophosphate.

In the present experiments the production of alpha-amylase and protease by *B. subtilis* BS(IV)1968 has been studied in culture media of different composition. We found that the biosynthesis of alpha-amylase and protease could be regulated by changing the components of the culture medium.

Materials and methods

Strain. *B. subtilis* BS(IV)1968 strain was used.

Culture media. R-19/3 inoculum medium: soybean meal, 1.2%; corn meal, 2.5%; corn steep liquor 2.4%; sodium chloride, 0.4%; calcium carbonate, 0.6%; palm oil, 0.5%; pH adjusted to 7.2 before sterilization.

BS-9n basal medium: starch, 4.8%; potassium chloride, 0.15%; magnesium sulphate (water-free), 0.02%; calcium chloride (water-free), 0.01%; calcium carbonate, 0.1%; ferrous sulphate, 0.001%; ammonium phosphate dibasic, 2.0%.

BS-9n/40 medium: BS-9n basal medium supplemented with 1.0% soybean meal.

BS-9n/41 medium: BS-9n basal medium supplemented with 1.0% soybean meal and 1.0% sodium citrate.

BS-9n/72 medium: BS-9n medium with 1.45% potassium biphosphate and 0.96% dibasic sodium phosphate instead of 2.0% dibasic ammonium phosphate.

In the first series of experiments, soybean meal, corn steep liquor, hazelnut meal and ground tomato seed (canning factory by-product), at concentrations of 0.5–1.0–1.5 and 2.0% respectively, supplied as protein source to the BS-9n basal medium. The pH of the media was adjusted to 7.2 before autoclaving.

Cultivation. The strain was grown by shaking at 28 °C in 100 ml or 6 litre volume of the above media dispensed in 500 ml Erlenmeyer flasks and 10 litre glass fermentor, respectively. Fermentor cultures were bubbled with 1 litre bacterium-free air per litre of medium and per minute, and stirred at 650–750 r.p.m. In both types of experiment the assays were performed in triplicate.

Inoculation methods. The inoculum medium was seeded with the spore suspension *B. subtilis* strain BS(IV)1968, by transferring 10^4 spores in one ml of the medium. The inoculum spore suspension contained 10^9 spores per ml.

The fermentation medium was inoculated with a 24-hour inoculum culture, using 10% (v/v) of the fermentation broth.

Enzyme activity assay methods. Enzyme activity determinations were carried out from centrifuged samples of the supernatant. For amylase activity determination the procedure of SMITH and ROE [13] was employed. Protease activity was measured with the method of Kaken Laboratories [14], the reagent was 1.2% casein solution in phosphate buffer (pH 7.38). Precipitating solution: acetic acid, 12.1 g; sodium acetate, 16.1 g; trichloroacetic acid, 16.3 g; distilled water, 1000 ml. Sodium carbonate solution: sodium carbonate, 4.24 g, in 100 ml distilled water. Phenol reagent [23].

Determination was carried out as follows. The mixture of 1 ml enzyme solution and 1 ml casein solution was incubated at 40 °C for 10 minutes, then 2 ml of precipitating solution was added. After incubation for 20 minutes the precipitate was filtered and 5 ml sodium carbonate solution and 1 ml phenol reagent were added to the filtrate. Incubation lasted for 20 minutes. The control was run as follows. To 1 ml enzyme solution 2 ml precipitating solution was added, the reaction mixture was kept at 40 °C for 10 minutes, and after the addition of 1 ml casein solution the mixture was incubated again. After filtration we proceeded as indicated above. Controls for the readings were performed by adding 1 ml casein and 2 ml precipitating solution to 1 ml phosphate buffer; the mixture was filtered and the above procedure was followed. The results were expressed according to the formula

$$\text{PUK/ml} = \text{dilution} \times (E_{\text{sample}} - E_{\text{control}}) \times 4.$$

Readings were taken at 660 μm in a Spectromom 360 spectrophotometer.

Reproducibility of enzyme activity assays for alpha-amylase was 100 ± 0.5 , for protease 100 ± 1.0 .

Reproducibility of experiments. The experiments were repeated five times. In the Tables and Figures, mean values for amylase and protease activities ($\bar{x}$, $\bar{y}$) are recorded. The greatest deviations are shown graphically, and standard errors of mean values are given numerically in the Figures. In the Tables, square values and standard errors of the mean are presented. For calculations the following formulae [22] were used:

$$\bar{x} = \text{mean enzyme activity} = \frac{\sum_{i=1}^n x_i}{n}; \quad n = 5$$

$$S_x = \text{square deviation} = \pm \sqrt{\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n-1}}; \quad n = 5$$

$$u_x = \text{standard error of mean} = \pm \frac{S_x}{\sqrt{n}}$$

The change of amylase and protease activity in various media was studied with the method of significant difference,

$$\Delta = \text{significant difference} \frac{\bar{x} - \bar{y}}{U_x^2 + U_y^2}.$$

The difference between activities obtained in different media was regarded as significant if the value of Δ was above 5 [22].

Results

1. *Effect of sodium citrate on amylase and protease production.* In the first series of experiments, amylase and protease production by *B. subtilis* strain BS(IV)1968 has been studied in the complex medium with and without sodium citrate. Results are indicated in Figs 1 and 2, and in Tables I and II. The shaded bars in the Figures represent the mean values ($\bar{x}$, $\bar{y}$) for amylase and protease activity in citrated medium, the white columns denote those in citrate free medium. The significant difference (Δ_1 , Δ_2) between the activities of amylase and protease obtained with quantitatively and qualitatively different protein sources is also presented. Values marked Δ_3 stand for the significant differences representing the enzyme activities found in the presence and absence of sodium citrate in media of identical protein content.

In Figure 1 and Table I it can be seen that amylase production did not depend on the quality and quantity of the protein sources. In citrated media containing different protein sources, maximum and minimum amylase activity was 223 U/ml and 184 U/ml, respectively. The significant difference between these activity values is 2.66; in citrate free media composed of various protein sources the highest amylase activity was 219 U/ml, and the lowest 196 U/ml. The corresponding significant difference was 2.08.

In the presence of sodium citrate the maximum yield of amylase is determined by the quantity of protein, the optimum protein concentration for amylase production ranging from 0.5 to 1.0%. In citrate free medium the same protein concentration was required for maximum production, except for the medium made with corn steep liquor; of this ingredient, 0.5% was the concentration ensuring maximum yield.

Media of qualitatively and quantitatively identical protein content with and without citrate, showed no significant difference in enzyme activity.

From Figure 2 and Table II it can be seen that the quality of proteins did not affect the production of protease. In media prepared with different protein sources and supplemented with sodium citrate, the significant difference between the highest and lowest protease activity readings (87 U/ml and 68 U/ml) was 1.36. If sodium citrate was omitted, the significant difference was 2.08, as calculated from the respective activity values of 209 U/ml and 143 U/ml.

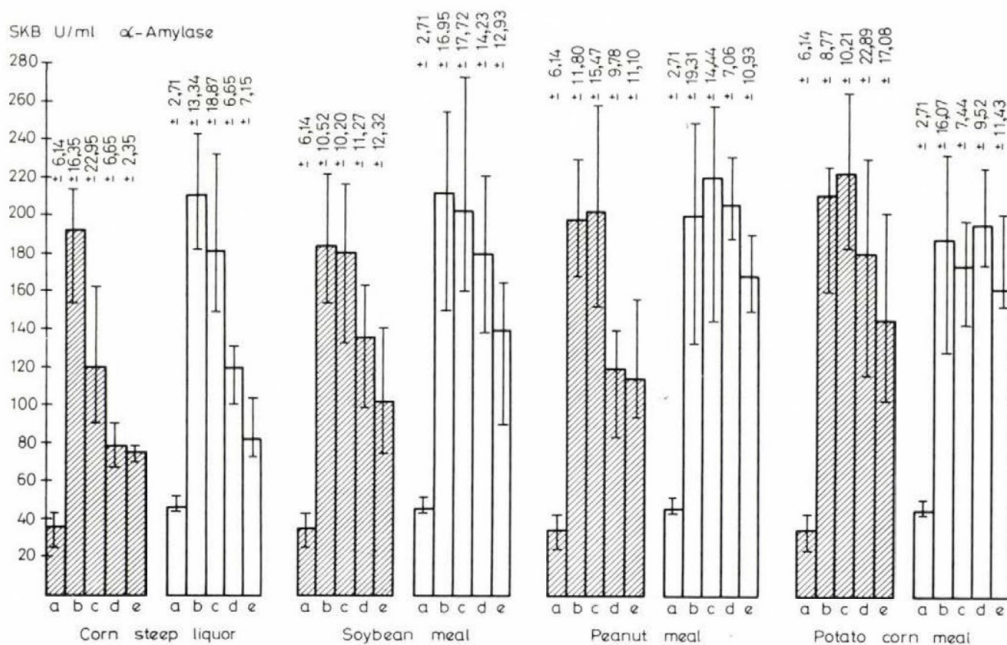


Fig. 1. Effect of sodium citrate on amylase production by strain BS(IV)1968. a = 0.0%, b = 0.5%, c = 1.0%, d = 1.5%, e = 2.0% protein content; = medium with sodium citrate; = medium without sodium citrate

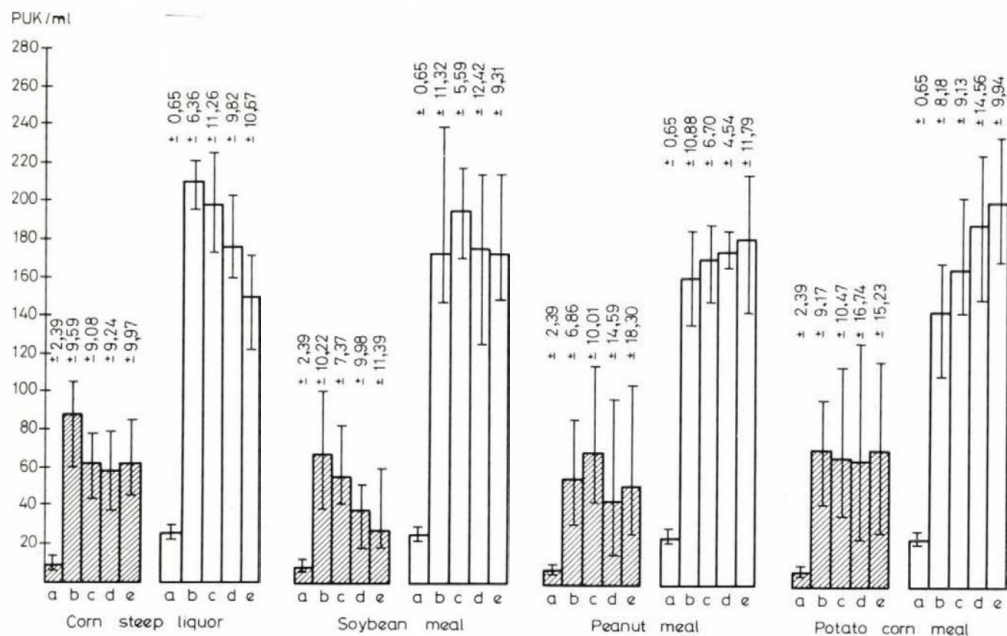


Fig. 2. Effect of sodium citrate on protease production by strain BS(IV)1968. a = 0.0%, b = 0.5%, c = 1.0%, d = 1.5%, e = 2.0% protein content; = medium with sodium citrate; = medium without sodium citrate

Table I
Effect of sodium citrate on amylase production by strain BS(IV)1968

Source of protein	Medium with Na-citrate					Medium without Na-citrate				Δ_3
	%	$\bar{x}$	S_x	U_x	Δ_1	$\bar{y}$	S_y	U_y	Δ_2	
Corn steep liquor	0.5	192	32.70	16.35	2.54 6.44 1.75 0.56	214	26.68	13.34	1.47 6.38 3.04 3.66	1.04
	1.0	120	39.75	22.95		180	37.75	18.87		2.01
	1.5	78	11.51	6.65		119	13.30	6.65		4.36
	2.0	74	4.06	2.35		83	14.29	7.15		1.18
Peanut meal	0.5	184	27.83	10.52	0.27 3.11 2.85 1.97 5.01	212	41.53	16.95	0.41 3.33 1.01 1.98	1.40
	1.0	180	27.09	10.24		202	46.88	17.72		1.07
	1.5	136	25.20	11.27		179	34.84	14.23		2.37
	2.0	103	27.53	12.32		141	31.69	12.93		2.13
Soybean meal	0.5	197	28.90	11.80	0.30 4.95 4.47 0.41 5.06	199	47.30	19.31	0.83 2.87 0.81 2.98	0.09
	1.0	203	40.93	15.47		219	38.20	14.44		0.76
	1.5	121	21.86	9.79		206	15.80	7.06		7.03
	2.0	115	24.83	11.10		167	24.40	10.93		3.34
Tomato corn meal	0.5	209	23.21	8.77	0.10 1.14 1.67 1.26 3.33	187	39.36	16.07	0.66 1.31 1.83 2.34	1.20
	1.0	223	27.01	10.21		174	19.68	7.44		3.88
	1.5	181	51.19	22.89		196	21.28	9.52		0.61
	2.0	145	37.97	17.08		161	28.00	11.43		0.78
	37	10.39	6.14			46	4.69	2.71		1.52

$\bar{x}, \bar{y}$ = mean enzyme activity; S_x, S_y = square deviation; U_x, U_y = standard error of mean; Δ_1, Δ_2 = significant difference between amylase activity values obtained with various amounts of protein sources, in media with and without sodium citrate; Δ_3 = significant difference in amylase activity between media with and without sodium citrate

Table II
Effect of sodium citrate on protease production by strain BS(IV)1968

Source of protein	Medium with Na-citrate					Medium without Na-citrate				Δ_3
	%	$\bar{x}$	S_x	U_x	Δ_1	$\bar{y}$	S_y	U_y	Δ_2	
Corn steep liquor	0.5	87	19.17	9.59	1.92	209	12.71	6.36	0.92	10.57
	1.0	61	18.16	9.08	2.25	197	22.53	11.26	1.40	9.37
	1.5	57	18.52	9.26	0.37	176	19.64	9.82	1.72	8.91
	2.0	62	19.94	9.97		151	21.34	10.67		6.08
Soybean meal	0.5	68	27.10	10.22	0.87	174	29.96	11.32	1.70	6.46
	1.0	57	16.48	7.37	1.61	195	12.52	5.59	1.39	15.22
	1.5	37	17.29	9.98	0.66	176	27.78	12.42	0.06	8.72
	2.0	27	22.78	11.39		175	22.79	9.31		10.60
Peanut meal	0.5	55	18.14	6.86	1.23	162	26.65	10.88	0.78	8.29
	1.0	70	26.49	10.01	1.53	172	16.40	6.70	0.37	8.47
	1.5	43	32.63	14.59	0.38	175	8.26	4.54	0.48	8.64
	2.0	53	36.59	18.30		181	28.89	11.79		5.92
Tomato corn meal	0.5	72	24.26	9.17	0.28	143	20.04	8.18	1.79	5.76
	1.0	68	27.71	10.47	0.05	165	22.36	9.13	1.51	6.95
	1.5	67	37.44	16.74	0.20	191	32.56	14.56	0.68	5.68
	2.0	72	34.05	15.23		203	24.34	9.94		7.20
	8.2		4.79	2.39		26	1.14	0.65		7.24

$\bar{x}$, $\bar{y}$ = mean enzyme activity; S_x , S_y = square deviation; U_x , U_y = standard errors of mean; Δ_1 , Δ_2 = significant difference between protease activity values obtained with various amounts of protein sources, in media with and without sodium citrate; Δ_3 = significant difference values between protease activity values obtained in media with and without sodium citrate

Protease production was not considerably affected by the amount of protein either in the presence or in the absence of sodium citrate; the significant difference was never more than 5.

A comparison of the protease activity in media with and without sodium citrate containing the same amount and quality of protein showed that protease production was definitely inferior in the presence of added citrate. A clear-cut difference was evident at all concentrations of each protein source; the Δ_3 value for the significant difference was in every case above 5.

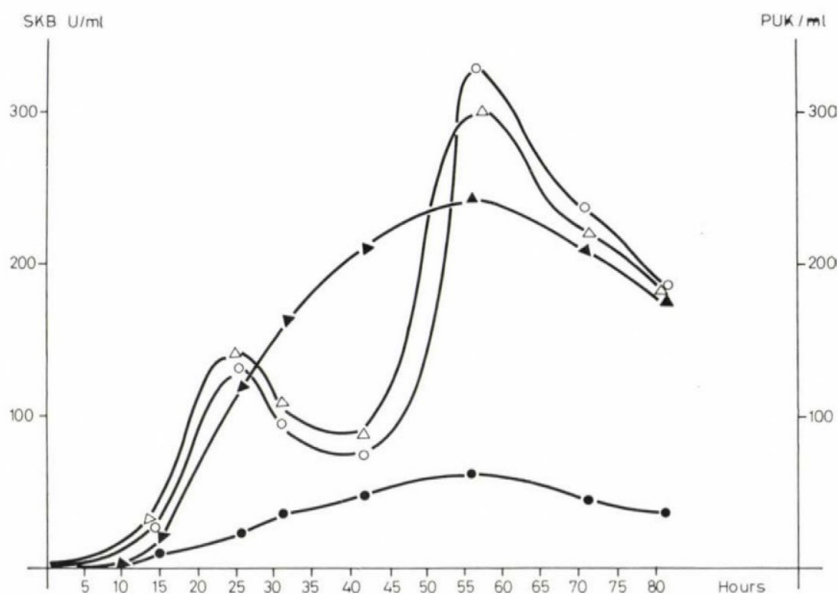


Fig. 3. Effect of ammonium salt on amylase and protease production. —▲—▲— = amylase production in medium containing ammonium salt; —●—●— = amylase production in medium without ammonium salt; —△—△— = protease production in medium containing ammonium salt; —○—○— = protease production in medium without ammonium salt

Our observations revealed that protease was not inactivated by sodium citrate.

2. In a second series of experiments we have studied the effect of ammonium salts on amylase and protease production on BS-9n/40 and BS-9n/72 media. As it is shown in Figure 3, enzyme production began in the 16th hour of cultivation. In the absence of ammonium salts, the amylase level was low during the whole cycle of fermentation; its peak activity, 62 U/ml, was read in the 57th hour of growth while in medium supplemented with ammonium salt the

maximum activity value was 240 U/ml. Protease activity was slightly influenced by the ammonium salt, the difference between yields in the presence and absence of ammonium being only about 10%.

Discussion

NOMURA *et al.* [16, 17] reported that from *B. subtilis* a factor stimulating amylase synthesis could be extracted by boiling. YOSHIKAWA and MARUO [18] demonstrated that this factor was an amine, and that putrescine and cadaverine were able to replace it in part. From the present studies one may suppose that ammonia was necessary for the synthesis of this amine. On the other hand, ammonia can act by promoting the synthesis of pyruvic acid decarboxylase, as suggested by HUBER [19] which latter hinders the accumulation of pyruvic acid, inhibiting thus amylase production. Several data indicate that sodium citrate promotes the formation of amylase, but its effect on protease synthesis has not been investigated. HUBER [19] also noted that the amylase activity in *B. subtilis* cultures can be increased by adding sodium citrate to the culture medium. He postulated that the mechanism of action of sodium citrate, decreasing the accumulation of pyruvic acid which inhibits amylase formation, is hindering phosphofructokinase activity, as a result of which the HMP pathway of carbohydrate breakdown assumes preponderance. Though there is some evidence suggesting that the cleavage of carbohydrates can take place *via* different pathways [20, 23], we rather assume that the influence of sodium citrate on protease production is exerted by affecting in some manner the sporulation process. We are planning further experiments in order to clarify this question and to explain the two peaks of the curve of protease production presented in Fig. 2.

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VITAMIN B₁₂ PRODUCING FERMENTATIONS WITH A MIXED BACTERIUM POPULATION OF SEWAGE SLUDGE ORIGIN

II. ANAEROBIC VITAMIN B₁₂ PRODUCING FERMENTATION IN ELASTIC FERMENTORS

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Summary. Vitamin B₁₂ production experiments in glass fermentors under pressure did not result in a higher yield. In closed, thin-walled rubber balloons (elastic fermentors) in which the maximum attainable pressure was 30—35 mm Hg, the yield was found to have increased. The permeability of the wall of the ELF to different gases is variable. The partial pressure difference results in the entrance of oxygen from the external air into the gas and fluid spaces of the ELF under pressure. This additional oxygen enhances the metabolism of non-vitamin-producing (microaerophilic) bacteria which are always present in the fermentation liquid and whose metabolic products are utilized by the anaerobic methanobacteria.

In a previous paper [1] a procedure employed for vitamin B₁₂ production from sewage sludge has been described. First the vitamin was produced by batch fermentation using a fermented sewage sludge containing medium. Since the introduction of Hungarian patent No. 153740, a bacterial population adapted to sludgeless medium and a semi-continuous procedure have been used. In the latter procedure, 10% of the fermentation liquid is removed daily and replaced by a medium described in the patent. One important component of the medium is methanol. On the addition of methanol, vigorous gas formation begins, the end product of which is in an ideal case 3 parts methane and 1 part carbon dioxide, according to the equation $4 \text{CH}_3\text{OH} \rightarrow 3 \text{CH}_4 + \text{CO}_2 + 2 \text{H}_2\text{O}$. The quantity of the produced gas changes with the amount of the methanol added. In industrial scale fermentations the gas produced daily is usually about two or three times the volume of the fermentation liquid. It has been shown that in the fermentation gas, the proportions of methane and carbon dioxide fairly approximate the ratio following from the above equation. The medium contains, however, a certain amount of sugar and ammonium hydrocarbonate, which also may contribute to the production of carbon dioxide during decomposition. The explanation lies close at hand that the amount of carbon dioxide released as a decomposition product of the latter medium constituents is negligible in comparison to the quantity of CO₂ formed from methanol. This simplified explanation seems, however, doubtful, if it is considered that

the methanobacteria are an important component of the fairly mixed bacterial population and, according to BARKER [2], they require carbon dioxide to maintain metabolic activity. According to GIOVANNI [3], methanobacteria are capable of utilizing carbon dioxide without solar energy. Further related data are to be found in the papers of LIEBMANN [4, 5]. Independently of the decomposition of methanol, a small amount of hydrogen sulphide is also produced during fermentation, the volume of which is negligible in comparison to that of the other gases.

Since in a given period of our investigations the high yields, obtained in the industrial scale fermentors, could not be reproduced in the small, 5–10 litre, laboratory-scale fermentors, it was supposed that bacterial metabolism may take a different course under the different conditions of hydrostatic pressure in the two types of fermentor. While in industrial-scale fermentors the pressure attributable to the high liquid column is more than 1 atm, in the small, 40 cm high laboratory fermentors it is only part of this value. It was found in aerobic fermentations that the production of certain metabolites improved if the pressure was slightly raised [6, 7]. We have therefore studied whether an increase in pressure would alter metabolic activity under the conditions of anaerobic fermentation, owing *e.g.* to a changed solubility of the gases utilized by one or the other of the bacteria present, or to an alteration of the degree of anaerobiosis. On the basis of these considerations, fermentation experiments were conducted on the one hand in glass fermentors under self-produced increased gas pressure, on the other hand in closed rubber balloon fermentors (elastic fermentors, ELF).

Since the B_{12} yield was soon found to be higher in ELFs than in laboratory scale glass fermentors used as control, metabolic activity in the ELF was investigated in more detail, with special regard to its easily measurable part, the gas metabolism.

Materials and methods

Bacteria. A mixed bacterial population originating from domestic sewage sludge and adapted to sewage sludgeless medium according to Hungarian Patent No. 153740, was used.

Fermentation system. (a) Anaerobic (oxidation–reduction potential, 400–600 millivolt), mixed bacterial, semi-continuous fermentation, pH 5.6–6.8, 30–32 °C. Ten per cent of the liquid was removed and substituted by fresh medium every 24 hours.

(b) Growth medium according to Hungarian Patent No. 153740 [1].

(c) Methanol added every 24 hours along with the fresh medium (0.4–1.2% of the total fermentor contents in the forthcoming experiments).

Elastic fermentor. Commercially available, unglued spherical rubber balloons, from possibly one and the same batch, in the uninflated state about 0.2–0.3 litre in capacity (producer, OGV-Palma). The balloons were furnished airtight with a perforated rubber stopper and a tap. Experiments were run with two litre fermentation liquid in a closed system. The gases formed during the 24-hour periods of fermentation were always removed prior to replacement of the medium. The volume of the filled ELFs, which had a fairly regular spherical shape, was calculated from their circumference.

Control fermentor. This was a 2-litre suction-bottle furnished with a perforated rubber stopper into which a glass tube with tap was inserted.

Measurement of the gas from the glass fermentor. The gas formed in the suction-bottle fermentor was collected in a graded glass cylinder under acidified (HCl) water, and measured. Gas production was plotted as a function of time and the total quantity of gas formed was determined by calculation of the area under the curve.

Assay of vitamin B₁₂ was performed as described [1].

Gas chromatographic measurements were carried out in a Carlo-Erba apparatus equipped with a heat conductivity detector, using H₂ as carrier gas.

Results and discussion

1. It was examined to what extent the values of actual gas production are in conformity with the expected values calculated from the equation described above [8]. Averages of numerous measurements performed with different quantities of added methanol showed that the actual gas production amounted to about 81% of the expected theoretical value, independently of the amount of methanol added. The remaining part of methanol, which apparently did not participate in gas production, was probably utilized as a carbon source. Isotope experiments are now in progress to substantiate this hypothesis.

2. Since the higher B₁₂ yield obtained in the industrial-scale fermentors had been attributed to the higher hydrostatic pressure present in these tanks, investigations into pressure relations were conducted partly in laboratory glass fermentors, partly in ELF's.

The higher gas pressure in the glass fermentors was due to the gas mixture formed from methanol. For this purpose, the outlet of the bottle was closed with a mercury column of changeable height, thus an overpressure up to 500 mm Hg could be adjusted according to choice. (Pressures above 500 mm Hg were not employed for safety reasons.)

Fermentations conducted under different pressures did not, however, result in higher than usual (control) vitamin B₁₂ yields; thus pressure differences in themselves did not account for the different laboratory and industrial scale production levels.

Parallel to pressure studies in glass-bottles, production experiments were also carried out in ELF's. The pressure to volume relations expectable in the scheduled range of volumes had previously been determined in empty ELF's. Results of such measurements are shown in Fig. 1. As seen the maximum pressure did not exceed 30 mm Hg in the applied range of volumes, indicating that the pressure in the bottom region of the industrial fermentor cannot be produced in an ELF. Another unexpected information emerging from Fig. 1 was the definite minimum of curves; this might have been in connection with the finer structure of the rubber material.

3. The first production experiments in ELF's had already led to the unexpected result that vitamin B₁₂ titres were higher than under similar con-

ditions in the glass fermentors used as control. The starting cultures, the composition of the medium, and the daily methanol doses were of course identical in the two kinds of fermentor. The first (A) set of columns of Fig. 2 compares the average production values measured in two semi-continuous ELF ferment-

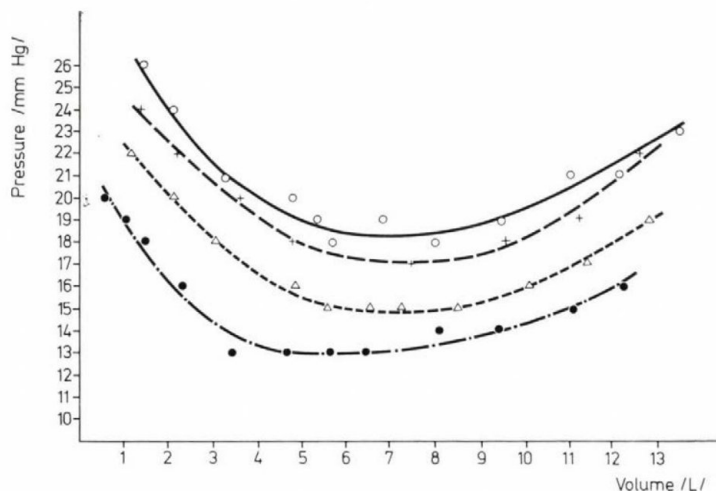


Fig. 1. Pressure—volume relations in ELF filled with air

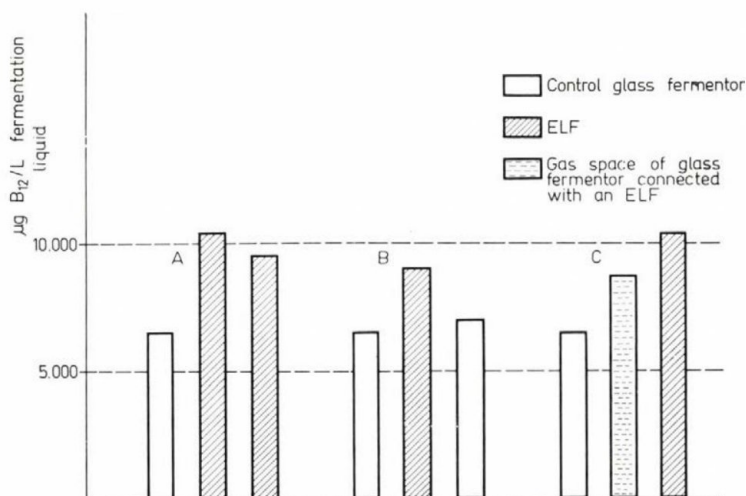


Fig. 2. Average production levels of vitamin B₁₂ obtained in different types of fermentors

tations over a period of several weeks with corresponding values for the glass fermentors used as controls. The values are always expressed in terms of μg cyanocobalamine per litre fermentation liquid. The height of the corresponding columns clearly shows that the vitamin B₁₂ yield was near to 50% higher in the ELFs than in the control glass fermentors.

4. To verify that the surplus vitamin production was actually related to the ELF, the yield of a semi-continuous glass-bottle control fermentation was transferred to an empty ELF and, after fermentation in the latter for several weeks, was returned to a glass fermentor. Comparison of yields measured over several weeks, as seen in the second (B) set of columns in Fig. 2, clearly indicated that yields in the ELF were definitely superior to both the previous and successive control productions.

5. In the above experiments, the difference in B₁₂ yield between laboratory and industrial scale fermentors had disappeared, but since production in the ELFs exceeded the industrial scale level, the production experiments in these were continued in order to elucidate the cause of the superiority of ELF-yields.

Since, however, fermentation in ELF cannot be realized on an industrial scale, the increase of yield was of a practical value only if the factors responsible for the improved production were identified and reproduced under industrial scale conditions.

To obtain more information on this problem, first the possible interaction between the rubber material of ELF and the fermentation liquid has been investigated. Production experiments were therefore conducted in glass fermentors connected with a rubber balloon under airtight conditions, so that the rubber matter of the balloon contacted the fermentation gases, but not the fermentation liquid. As can be seen from the third (C) set of columns in Fig. 2, fermentation in this kind of a "hybrid" fermentor over a period of several weeks led to a higher average B₁₂ vitamin yield as compared with the control glass fermentor culture, but did not attain the level measured in the corresponding ELF system.

Even in several weeks, no increase of yield over the control level was obtained when a rubber balloon cut into pieces was placed in the fermentation liquid processed in a glass fermentor.

Thus, the interaction between the fermentation liquid (*viz.* fermentation gases) and the rubber material of the ELF was not in itself responsible for the increase of vitamin yield, although an effect of this kind obviously did exist under the given conditions.

6. The oxidation-reduction potential and the pH of the fermentation liquid were continuously recorded in the ELFs. The measurements during the several months experimental period did not differ from the mean values established for several years (see Materials and methods), thus these parameters did not account for the higher production yields.

7. Further examinations were conducted to clarify whether or not the gas spaces of the two kinds of fermentor differed in a qualitative or quantitative respect and whether an eventual difference could have affected the fermentation. The quantity of gas present in the ELF 24 hours after setup was

therefore compared with the total volume of gas formed in the control glass fermentor in an identical period of time. The results were correlated with the theoretical gas production values deduced from the quantity of added methanol. It was found that whilst the glass fermentors produced about 80% of the theoretically expected gas volume (see item 1), the ELF's contained only 40–50% of the same in 24 hours. There are two possible explanations of this problem:

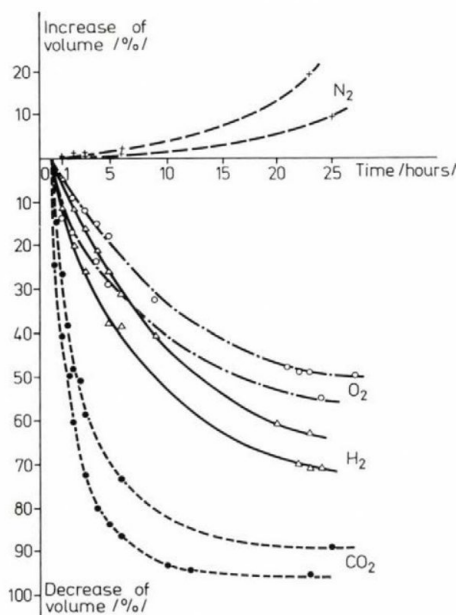


Fig. 3. Volume changes of ELF's filled with different gases

either gas production was less in the ELF, or part of the gases produced had escaped across the wall of the rubber balloon. Since the latter explanation seemed more probable, the possibility of gas escape has been examined in more detail.

8. Empty ELF's were filled with the gases under study (H_2 , N_2 , O_2 , CO_2 , CH_4) and the changes in volume taking place in 24 hours were measured. The results are shown in Fig. 3. The pair of curves presented for each gas represents the limit values for a number of measurements. Curves for methane are not shown in the Figure, on the one hand because they fell into the oxygen region, and on the other because the methane used was not a commercially available pure preparation, but a fermentor gas purified from carbon dioxide and sulphur hydrogen.

The curves clearly indicate that only the nitrogen-containing balloon increased in volume during the period of measurement. All other gases were capable of penetrating across the rubber wall, and quantities of them escaped

from the balloon. The differences in penetrating capacity ("escape velocity") cannot be explained by motility, molecular weight and molecular diameter. The difference in "escape velocity" of carbon dioxide and hydrogen is particularly intriguing, because on the basis of the above properties, hydrogen might have been expected to escape at the highest rate.

Of the gases studied, the behaviour of nitrogen deserved special consideration. The gas chromatographic analysis of the slight increase of volume observed with this gas in 24 hours revealed that nitrogen escaped from the balloon at a very slow rate, but oxygen from the external air entered into the balloon against pressure (Fig. 4). In our opinion, this phenomenon was due to

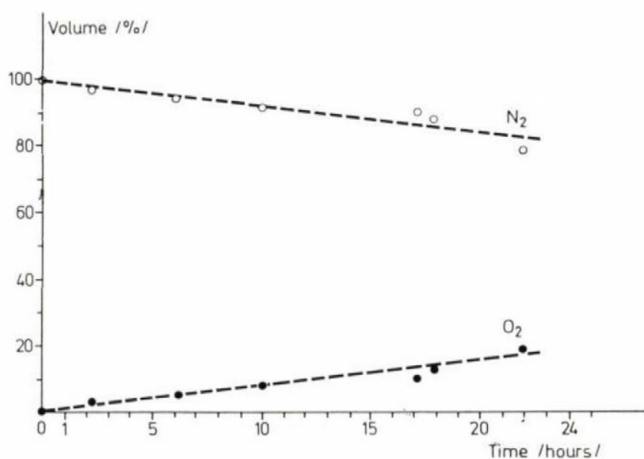


Fig. 4. Changes of gas composition in nitrogen-filled ELF

the considerable difference between the partial pressures on the two surfaces of the rubber membrane. Penetration of oxygen across the wall of the balloon originally filled with nitrogen lasted until the two gases had attained the proportions characteristic of air, *viz.* until the difference between the partial pressures had disappeared.

These findings put the interpretation of the "escape curves" into a different light, since each curve represents in fact the resultant of gas escape and oxygen penetration, as reflected by the volume changes of the corresponding gas. The curve for the oxygen-filled balloon, however, signifies escape alone, as presumably no penetration took place in this particular case. Differences in partial pressure cause, of course, also air nitrogen to penetrate the balloon, but the movement velocity of this gas is so low that the quantity entering the balloon within the period of measurement is negligible.

To confirm that with different partial pressures gas migration is a general phenomenon, a balloon was filled with air up to its own volume (about 0.3

litres) and placed in a carbon dioxide atmosphere. The penetrating carbon dioxide caused the balloon's volume to increase considerably in a single hour, followed by a multiple increase by the end of 24 hours.

9. Based on the above findings, changes of the gas space of an ELF containing the fermentation liquid were measured by gas chromatography for 24 hours following the addition of methanol. As can be seen from the curves presented in Fig. 5, the following events took place during this period. From methanol, the bacterial population produced methane and carbon dioxide. These gases tended to escape across the rubber wall at individual rates, while

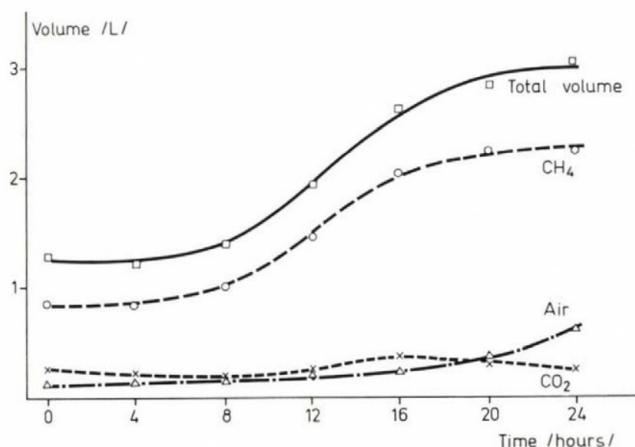


Fig. 5. Changes of gas composition in ELF during fermentation

air gases (chiefly oxygen) entered into the gas space of the ELF, compensating the reduction of volume caused by the escape of the fermentation gases. The observation that ELFs contained less fermentation gases than the control glass fermentors after 24 hours, was actually the resultant of the two oppositely oriented gas migrations. At 24 hours after the addition of methanol, the gas content of the ELF was different because gas migration from outside occurred throughout the whole period, whereas the production of methane and carbon dioxide took different times depending on the microbiological composition of the population, even if the amount of added methanol was kept constant. Differences in the duration of methane and carbon dioxide production considerably influenced the final quantity of gas present inside the ELF.

Analysis of the composition of fermentation gas in ELFs revealed the following differences from the control fermentor gas.

(a) The original methane : carbon dioxide ratio (3 : 1) varied in the ELF, owing to the more rapid escape of the latter gas;

(b) differences in partial pressure caused oxygen to enter from the surrounding air across the rubber wall into the gas space of the ELF.

10. The penetration of oxygen into the gas space of the ELF is especially relevant as this phenomenon cannot take place in a glass fermentor. There is reason to suppose that the oxygen diffusing into the gas space of the ELF is dissolved in the fermentation brew with which it is in contact. On the other hand, there is evidence that oxygen crossing the rubber wall enters directly into the fermentation liquid. When examined after a few weeks of fermentation, the liquid-contacting area of the inner rubber wall was covered by a several millimeter thick, dense, adherent mass of well sporulating hyphomycetes [9]. The fungus, identified as a member of the genus *Graphium*, was found to be an obligatory aerobic organism. Its growth ability at the highly negative oxidation-reduction potential of the anaerobic culture fluid seemed surprising until it was discovered that the fungus grew exclusively over the inner surface of the rubber wall exposed to oxygen. It follows that at the sites of oxygen penetration, the fungi were able to utilize for growth the nutrients present in the fermentation liquid, probably methane as well. Absence of fungal growth in the bottom area of the ELF resting on the tray indicates that no penetration of oxygen could take place in this region.

Mention has already been made of the fact that the sewage-derived mixed bacterial populations represent a septic fermentation subject to further contamination. Thus, on culturing on solid medium, large numbers and many types of aerobic and microaerophilic bacteria are encountered. These accompanying organisms do not die off under the given conditions of anaerobic fermentation. LIEBMANN [4] holds the view that the utilization of organic compounds, under methane and carbon dioxide release by methanobacteria, requires the presence of facultatively anaerobic or microaerophilic bacteria capable of synthesizing fatty acids and alcohols, *viz.* compounds which can be utilized by the methanobacteria. The microaerophilic organisms require in turn a small amount of oxygen.

The important role of aerobic and facultatively aerobic organisms in the survival of anaerobes both in mixed cultures and in nature has been pointed out by SMITH [10], who referred to ROSEBURY's statement [11] that in the oral cavity, anaerobes are thirty times more numerous than aerobes and are very sensitive to oxygen. In the ELF, the presence, growth and metabolic products of non-anaerobes, above all of microaerophilic bacteria, are to be sought chiefly next to the wall. These substances are important either for anaerobic (B₁₂-producer) growth, or are themselves precursors of vitamin B₁₂; in either case, their presence may account for the greater vitamin B₁₂ yields found in ELFs.

Microbiological analysis of fermentor populations is a difficult task. Many anaerobic culturing experiments performed in this laboratory have failed especially in respect of the isolation of the important methane-producing, Gram positive cocci and diplococci. These microorganisms belong probably to

the group of the extremely oxygen-sensitive ("fastidious") anaerobes, whose isolation requires prereduced media and a carbon dioxide or nitrogen atmosphere free of oxygen [12]. These technical difficulties have prevented us from the substantiation of our hypothesis concerning the higher vitamin B₁₂ yields in the ELF. This work would namely require pure cultures of the anaerobic and microaerophilic bacteria present in the fermentation liquid and their simultaneous inoculation into the medium at appropriate proportions.

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EFFECT OF DDT AND SEVIN ON SOIL FUNGI

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Summary. Both DDT and Sevin completely inhibited *Alternaria humicola*, *Trichoderma viride*, *Botryotrichum* sp., *Helminthosporium sativum*, *Sepedonium* sp., *Papularia* sp. In addition, DDT inhibited *Mucor* sp., *Hormiscium* sp., *Stachybotrys atra*, *Rhizoctonia solani*, *Synsporium* sp., *Gliocladium sagariensis*, *Botrytis* sp., *Myrothecium stratisporum*, *Phoma humicola* and *Macrophoma* sp. which on the other hand were stimulated by Sevin. The number of *Curvularia lunata*, *Fusarium chlamydosporum*, *Mortierella rammaniana* was reduced by both insecticides. However, they enhanced the population of *Aspergillus* spp., *Paecilomyces* spp. and *Acrophialophora* sp. The addition of F. Y. M. to soil counteracted the depressing effect of DDT and increased the soil fungal population moderately. Sevin failed to bring about any considerable modification in the fungal population even at higher doses whereas at lower doses the insecticide stimulated it to some extent.

The use of pesticides for controlling plant pests is one of the assets of modern agriculture for higher crop yields. Besides controlling specific pests, pesticides in soil may temporarily or permanently hamper the biodynamics involved in soil fertility, through their direct and/or side effects [1—3]. The chlorinated hydrocarbon insecticides like DDT which persist in soil longer were found to be detrimental to biological processes since higher doses and their accumulated residues inhibit soil respiration [1, 4—8] and nitrification [1, 9—13]. Carbamate insecticides like Sevin were used as an alternative to DDT, since they carry less hazards and are less persistent [14].

Nematodes, fungi and nitrifying organisms are readily inhibited and/or killed by pesticides unlike spore forming bacteria and occasionally actinomycetes which show a relatively more tolerance [15]. Nitrifying organisms may be retarded by a few days to three or four months whereas fungi may return to normal quickly or slowly [17, 18].

Pesticides often affect non-parasitic soil organisms by killing and/or reducing the population of one or more forms. The lethality depends on the chemical, its doses, soil properties, nature of organisms, and various environmental factors [15, 16, 19, 20].

The present studies were undertaken to investigate the effect of DDT and Sevin on the quantitative and qualitative changes of soil fungi.

Materials and methods

Sandy loam alluvial soil from 0–23 cm layer of IARI Farm, New Delhi, was air dried, lightly powdered and sieved through a 2 mm sieve. Four hundred grams of soil were taken in each plastic container. Sevin and DDT (technical grade) were added separately at varying rates ranging from 1 to 1000 ppm and the effect of Sevin was also examined at a rate of 5000 ppm. One per cent organic matter as farmyard manure was also applied to soil in the case of DDT at 50 ppm and at higher concentrations. All the components were mixed thoroughly and moisture was maintained at 1/3 of the water holding capacity of the soil. Soil samples were drawn at periodic intervals for fungal count. Total fungal counting was done on Martin's rose Bengal agar medium. Fungal colonies were isolated from Petri dishes maintained on PDA. Most of them were identified up to the species level [21, 22].

Results

Twenty-four to twenty-five and six to seven genera of fungi were recovered in untreated (control) and insecticide treated soil, respectively. In treated soil, *Aspergillus* spp. alone constituted the bulk (about 50%) while species of *Curvularia*, *Penicillium*, *Mortierella*, *Paecilomyces*, *Acrophialophora*, *Rhizopus* and other constituted the remaining fungal population.

When the effects of DDT and Sevin (Fig. 1) on soil fungal population are compared it is revealed that DDT is more toxic than Sevin. The former

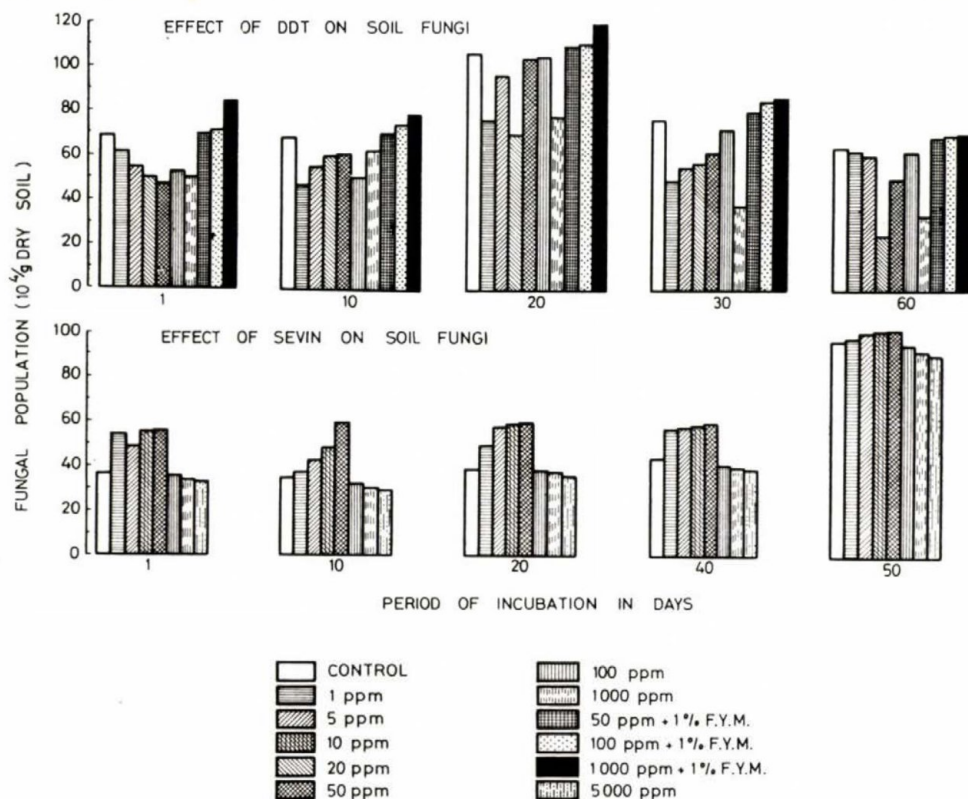


Fig. 1. Effect of DDT and Sevin on soil fungi

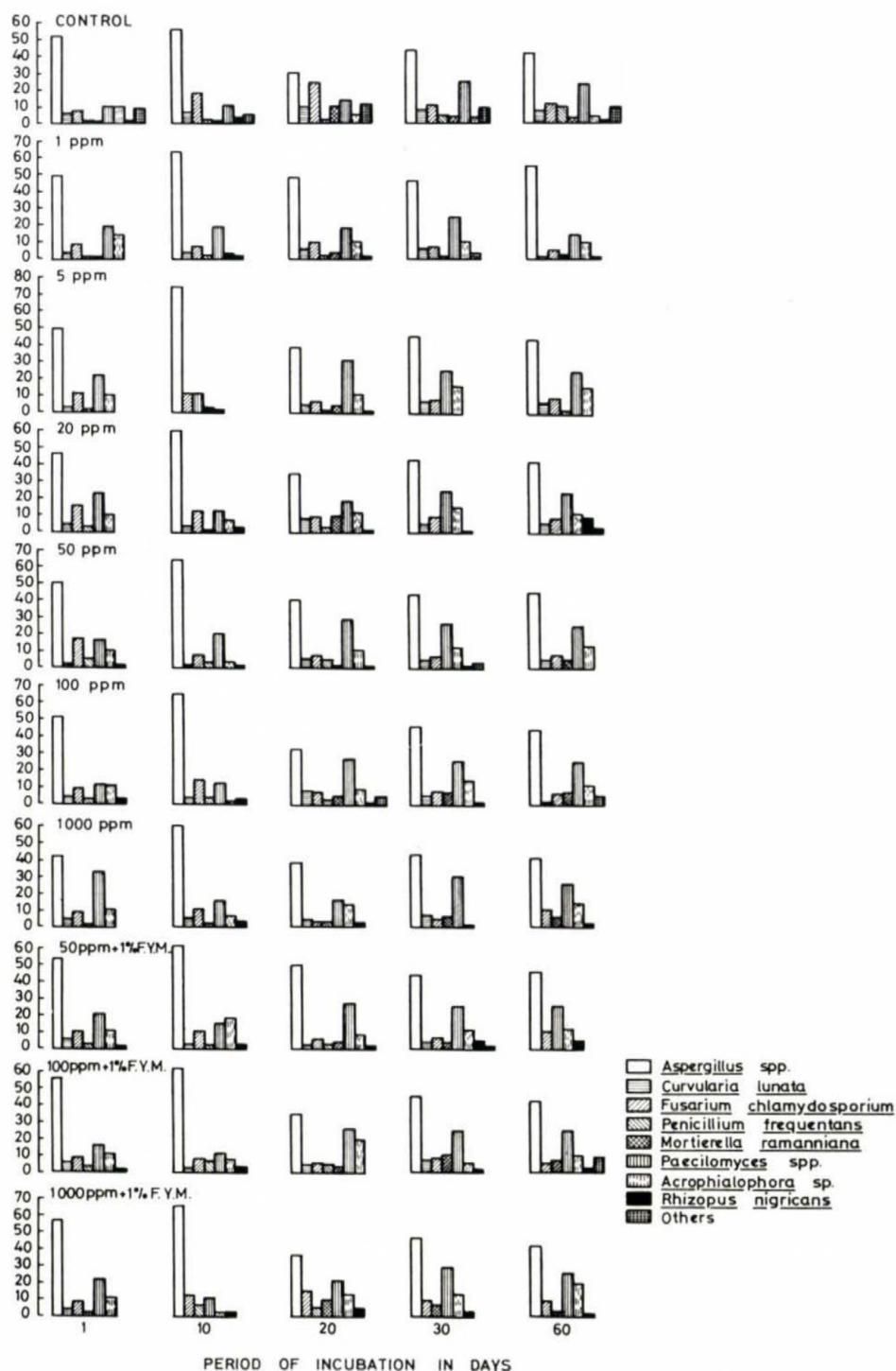


Fig. 2. Effect of DDT on incidence of soil fungi

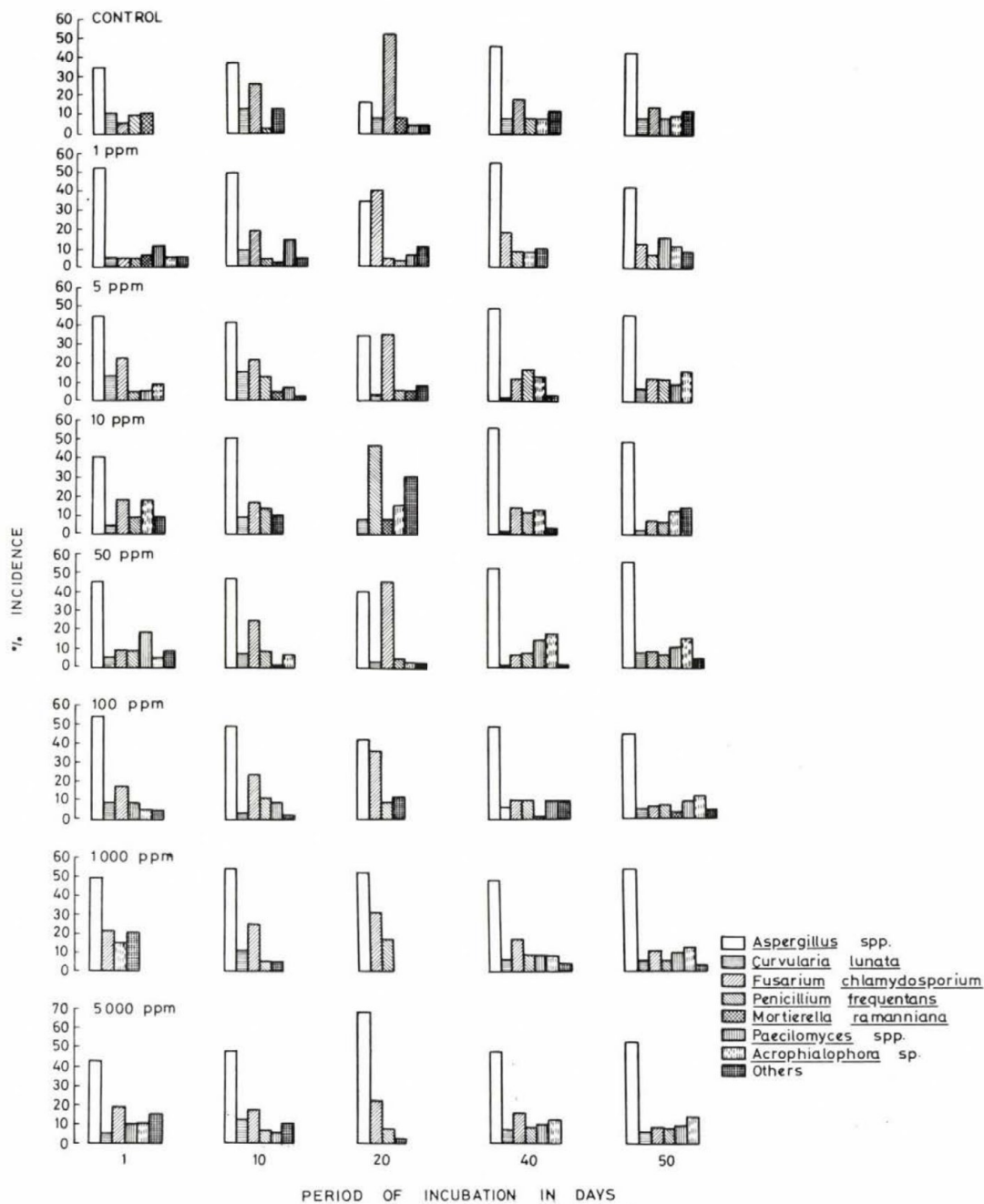


Fig. 3. Effect of Sevin on incidence of soil fungi

brought about more modifications in the population by reducing their number in all the treatments except where farmyard manure was applied. The depressing effect of DDT seemed to be counteracted by farmyard manure. Even at higher doses, Sevin could not bring about a considerable modification in the population. With lower doses of Sevin the total number of fungi increased to some extent while it decreased moderately at higher levels.

DDT in most cases and Sevin in all cases stimulated *Aspergillus* spp., viz. *A. terreus*, *A. nidulans*, *A. flavus*, *A. fumigatus* (Figs 2 and 3). However, it was decreased with DDT on the first day at every level except with farmyard manure amended soil where it was rather augmented. *Curvularia lunata* decreased in DDT treated soil as well as at all the levels of Sevin except at 5 ppm where it increased slightly on the 1st and 10th days. *Fusarium chlamydosporum* increased on the first day with different doses of DDT but decreased during progressive incubation, whereas Sevin decreased its population at all the levels and moreover it was not detected in 10 ppm on the 20th day. *Penicillium frequentans* increased in DDT treated soils up to the 20th day but further incubation resulted in a decrease and subsequent disappearance of the fungi. At various levels of Sevin, the population was decreased on the first day and was not detected at all in 100 to 5000 ppm, but increased with further incubation. In DDT treated soil *Mortierella rammaniana* was not detected on the 1st, 10th and 30th days; at 1 to 50 ppm, it showed a decrease on the 20th day but was increased at 100 and 1000 ppm with and without organic matter on the 30th and the 60th days. With Sevin, it disappeared except at lower levels (1, 5, 10, 50 ppm) where it appeared on the 10th and 20th day in decreasing order. *Paecilomyces* spp. viz. *P. varioti*, *P. divaricatum* and *Acrophialophora* sp. increased at all levels of DDT, with DDT plus organic matter treatment, and Sevin. However, at 10 ppm of Sevin no species of *Paecilomyces* were detected. *Rhizopus nigricans* showed an increase with DDT.

The results indicated that DDT was more detrimental to fungal population than Sevin. Both DDT and Sevin inhibited *Alternaria humicola*, *Trichoderma viride*, *Botryotrichum* sp., *Helminthosporium sativum*, *Sepedonium* sp., *Papularia* sp. However, in addition, DDT inhibited *Mucor* sp., *Hormiscium* sp., *Stachybotrys atra*, *Rhizoctonia solani*, *Synsporium* sp., *Gliocladium sagariensis*, *Botrytis* sp., *Myrothecium stratisporum*, *Phoma humicola* and *Macrophoma* sp. which, on the other hand, were stimulated by Sevin.

Discussion

DDT and Sevin affected soil fungal population at varying rates as some species of fungi were more affected by DDT and Sevin than others. The affected ones disappeared completely whereas the others showed a fluctuation in their incidence. This may have been caused by a single factor or due to the

cumulative action of several factors. The stimulatory effect of DDT and Sevin on the incidence of *Aspergillus* spp., *Paecilomyces* spp. and *Acrophialophora* sp. might have been due to their effect on the environment making it less competitive by killing some forms [23], and/or by the utilization of dead cells [24] and/or by transformation of residual fragment [25, 26] of pesticide as a possible source of readily available substrate.

The total number of fungi was increased or decreased with DDT and Sevin although the number of species was reduced appreciably. Such findings were reported also by other workers [16, 20, 21]. *Trichoderma viride* which was detected in the untreated soil (control) was altogether inhibited in the insecticide treated soil. The dominance of certain species of fungi in treated soil may be due to their rapid growth, higher tolerance to the chemicals, or their capacity to restoration. The inhibition of species of *Alternaria*, *Helminthosporium* and *Sepedonium* by Sevin and *Rhizoctonia*, *Botrytis*, *Myrothecium*, *Phoma* and *Macrophoma* by DDT from treated soil is important as they may be parasitic, depending on the availability of suitable hosts.

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IMMUNOGENICITY OF SMALLPOX VACCINES PREPARED FROM STRAINS OF DIFFERENT REACTOGENICITY

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Summary. Smallpox vaccine prepared from the Budapest strain, the strain used for vaccine production in Hungary till 1969, has been compared with a Lister strain vaccine by measuring the neutralizing antibody response to primary vaccination of children, and in the active mouse-protection test. The Lister strain vaccine induced a significantly larger neutralizing-antibody response than did the Budapest strain vaccine. The mouse-protection test, on the other hand, did not show a significant difference between the two strains. The good immunogenicity and the low reactogenicity of the Lister strain justify its introduction in vaccine production in Hungary.

The virus strains used in smallpox vaccine production vary in virulence for animals and reactogenicity in man [1–9]. Furthermore, it has been suspected for several decades that vaccines of different origin vary in protective capacity against smallpox [10]. This suspicion, not yet investigated by epidemiological methods, has been confirmed by several workers [11–13] in experimental animals. Furthermore, it was claimed that the intensity of the antibody response [9] and the resistance to revaccination were dependent on the vaccine strain [14].

The majority of the countries free of smallpox, those having maintained the compulsory vaccination system, have made efforts to reduce the reactogenicity of their smallpox vaccine. However, the reduced reactogenicity must not be accompanied by a reduced immunogenicity, a phenomenon often experienced with other live viral vaccines. Apparently, there is no well-defined correlation between these two parameters, as the highly protective vaccine prepared from the Lister strain is one of the least reactogenic smallpox vaccines.

In Hungary, the smallpox vaccine used for both primary and revaccination had been prepared from the Budapest strain. Subsequently, it was shown that the vaccine prepared from the Lister strain was less reactogenic in children and less virulent for animals than the Budapest strain vaccine [15–16]. Relying upon these results, in 1969 the Lister strain has been introduced for compulsory primary vaccination.

We have failed in finding any difference in the postvaccination neutralizing antibody titre of revaccinees when the Budapest strain vaccine was compared with the Lister strain vaccine in small groups of volunteers [17]. Since

the antibody response to primary vaccination is a more reliable indicator of the efficacy of vaccination than that to revaccination, in the present study we have examined the serological response of primovaccines. In addition, we have studied the protection against neurovaccinal challenge of animals immunized with the Lister, Budapest and some other vaccine strains.

Strictly speaking, we wished to know how the introduction of the less reactogenic Lister strain influenced the effectiveness of smallpox vaccination.

Material and methods

(1) Primary vaccination of children

Vaccines. Glycerolated dermovaccines (Human, Budapest) were prepared from the Lister and Budapest strains. The virus titre of the two vaccines was 1.3×10^8 and 1.04×10^8 PFU/ml, respectively, as measured on the chick chorioallantoic membrane (CAM).

Children. One-year-old children eligible for primary vaccination were grouped at random.

Vaccination. All children were vaccinated by scarification at the same place and time.

Sampling. Blood was taken from the finger pad three weeks after successful vaccination (major reaction). The blood drop was immediately diluted at a given rate in an anticoagulant solution. The blood plasma samples were stored in the frozen state until subjected to neutralization (NE) test carried out as published earlier [17]. For the comparison of the geometric means (GM) of titres, Student's two-sample *t* test was used.

(2) Experiments in mice

Vaccines. Glycerolated or lyophilized dermovaccines (Human, Budapest) prepared from the Lister, Budapest and EM-63 strains and a 20% suspension of CAMs inoculated with the lyophilized dermovaccine of the Wyeth Vaccine Institute (kindly supplied by the Smallpox Eradication Unit of the WHO). Prior to each active immunization experiment, the vaccines were titrated on CAM.

Active immunization. Swiss mice from the breeding of the National Institute of Public Health, Budapest, were used. Two groups, each consisting of 10 to 20 mice, were inoculated with each vaccine intraperitoneally with inocula differing tenfold in virus content. The volume of the single inoculum was 0.5 ml throughout. The groups receiving the same pock count of different vaccines were inoculated simultaneously.

Challenge. The immunized groups were challenged intracerebrally with the IHD (International Health Division) strain of vaccinia virus on the 14th postvaccination day. The strain was stored in CAMs and, some days before the challenge, subjected to an intracerebral passage in mice. The suspension to be used as inoculum was prepared on the day of challenge in 1.5 ml PBS/brain. The suspension was centrifuged, and the 20-fold diluted supernatant was used as inoculum (0.03 ml/mouse). Controls were challenged with the above challenge dose and its tenfold dilution series. The observation period was 14 days. Death occurring 3 to 14 days after challenge was regarded specific if it had been preceded by symptoms characteristic of IHD virus infection. The vaccine dose protecting 50% of the challenged animals (ED_{50}) and the LD_{50} of the inoculum were calculated by the Reed-Muench formula [18]. The experiment in which all the vaccines under study had been examined simultaneously was evaluated by probit analysis.

Results

(1) Primary vaccination of children

In Table I, the data for 29 and 27 children primovaccinated with the Lister strain and Budapest strain vaccine, respectively, are shown. The GM

calculated from the NE titres for the reactors of the Lister group was significantly higher than that calculated for the reactors of the Budapest group ($t_{50} = 2.080$, $P < 0.05$).

(2) *Simultaneous experiments in mice with vaccines prepared from different strains*

(a) Vaccine strains Lister and Budapest. The results of comparative mouse-protection experiments are shown in Fig. 1. The challenge dose ranged between 100 and 320 LD₅₀. A linear relation was found between the log dose of vaccine and survival in per cent. Part of the repeated experiments covered each other, *viz.*, the largest ED₅₀ (258 PFU) calculated for the Lister strain vaccine fell in the range of the data for the Budapest strain vaccine. There was no appreciable difference in immunogenicity between the two vaccines.

Table I

Distribution by NE titre of children successfully primovaccinated with the Lister or the Budapest strain three weeks previously

Vaccine strain	No. of vaccinees tested	Distribution by NE titre			
		<1:4	1:4	1:16	1:64
Lister	29	2	6	16	5
Budapest	27	2	10	15	—

(b) Simultaneous experiments with the Lister, Budapest, Wyeth and EM-63 vaccines. The mouse-protective value of the vaccines prepared from the Lister, Wyeth and EM-63 strains was comparatively studied in three repeated experiments (Fig. 2). Since the challenge dose was larger than in experiment (a), the ED₅₀ of the Lister strain vaccine was also larger.

The ED₅₀ of the Wyeth strain vaccine was more than twice, that of the EM-63 strain vaccine was more than five times as large as that of the Lister vaccine. The ED₅₀ values obtained in different experiments did not cover each other; the smallest ED₅₀ dose of the Wyeth strain vaccine just approximated, that of the EM-63 strain vaccine definitely exceeded the highest value established for the Lister strain vaccine. The order of the vaccines beginning with the least effective one was: EM-63, Wyeth and Lister.

In Fig. 2 data for the single experiment in which the Budapest strain was included, are marked with circles. To evaluate this experiment, probit transformation of the percentage survival was applied. The relation between the logarithms of the vaccine doses and the probit values was linear for each of the vaccines. The regression coefficient calculated for the EM-63 strain vaccine was the highest, that for the Lister strain vaccine was the lowest; the difference

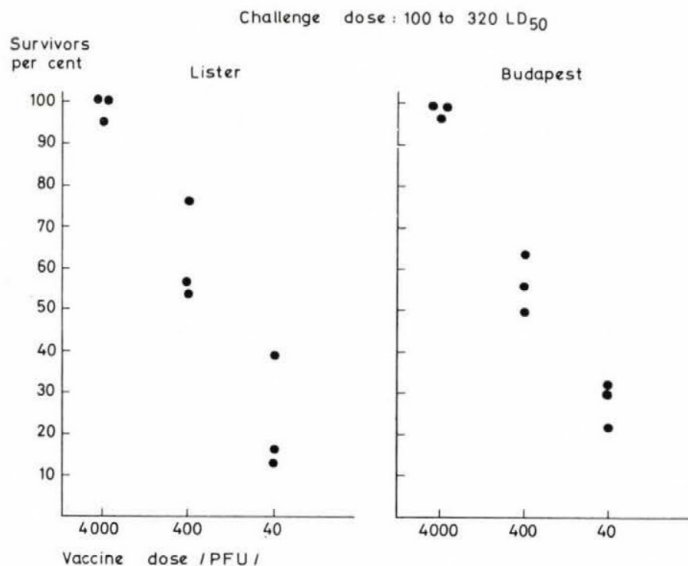


Fig. 1. Relationship between immunizing vaccine dose and percentage of animals surviving the challenge. Vaccine strains, Lister and Budapest. ED₅₀ values of three individual experiments: *Exp. 1.* Lister 85, Budapest 145; *Exp. 2.* Lister 258, Budapest 196; *Exp. 3.* Lister 220, Budapest 296. Mean of three experiments: Lister 187, Budapest 212

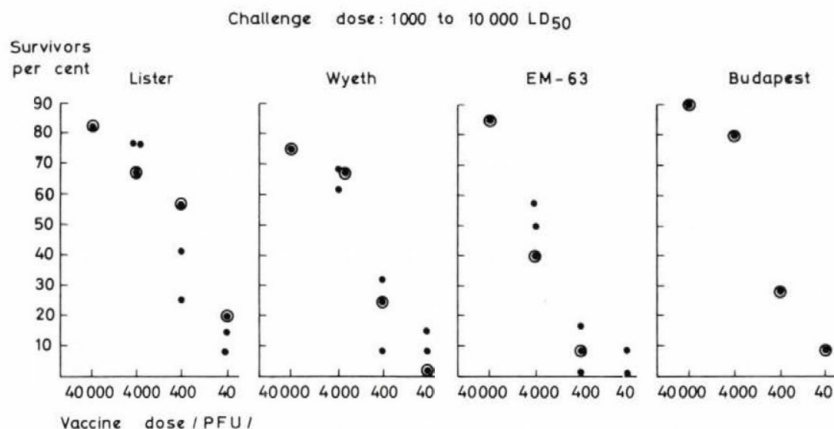


Fig. 2. Relationship between immunizing dose of vaccine and percentage of animals surviving the challenge. Ringed dots indicate values calculated by probit analysis. Vaccine strains: Lister, Wyeth, EM-63 and Budapest. ED₅₀ values of three individual experiments: *Exp. 1.* Lister 645, Wyeth 1081, EM-63 3305; *Exp. 2.* Lister 976, Wyeth 1740, EM-63 approx. 4000 (none of the animals immunized with 400 or 40 PFU survived); *Exp. 3.* Lister 551, Wyeth 2298, EM-63 4444, Budapest 1110. Mean of three experiments: Lister 724, Wyeth 1706, EM-63 3872

between these extreme values was at the limit of significance. The regression coefficient for the remaining two vaccines was approximately identical and did not differ significantly from the others. The ED_{50} of the Lister strain vaccine was significantly smaller than those of the Wyeth and the EM-63 vaccines, but did not differ significantly from that of the Budapest strain vaccine, which was significantly smaller than that of the EM-63 strain vaccine. The results of the calculations are presented in Tables II and III.

Table II*Regression coefficients and regression equations for log dose*

	Vaccine strain	
	Budapest	Lister
Regression coefficient (R.C.)	0.926	0.597
Scattering of R.C.	0.235	0.184
Regression equation	$Y = 0.926x + 2.218$	$Y = 0.597x + 3.541$
	Vaccine strain	
	Wyeth	EM-63
Regression coefficient (R.C.)	0.826	1.241
Scattering of R.C.	0.202	0.302
Regression equation	$Y = 0.826x + 2.139$	$Y = 1.241x + 0.292$

Table III *ED_{50} values and their fiducial limits*

	Vaccine strain			
	Budapest	Lister	Wyeth	EM-63
ED_{50}	1012	289	2896	6226
Fiducial limits	lower	330	64	743
	upper	3090	1312	10 120
				20 320

Taking into consideration the graphical illustration, the calculation with the Reed-Muench formula as well as the results of the probit analysis, we may state that (i) in the active mouse-protection test there exists a linear relationship between the logarithm of the immunizing dose and the percentage of survivors; (ii) the dose-response curves ran parallel — a lack of parallelism could be supposed only in the Lister/EM-63 relation, but the difference did not surpass the level of significance; (iii) the order of the vaccines on the basis of increasing immunogenicity was EM-63, Wyeth, Budapest, and Lister.

Discussion

The reliability of the experimental methods applied in the present comparative study is to some extent questionable. Changes in the NE titre provide an acceptable indicator of the fluctuation of protection, but the supposition that the higher the postvaccination titre the more protective the vaccine against smallpox, has no epidemiological support. The value of our mouse-protection test is also questionable. At present, determination of the ED₅₀ is the most accepted method for measuring the immunogenicity of vaccines and, according to the above calculation, the mouse test is suitable for this purpose. For the smallpox vaccine, there is, however, no epidemiological evidence to verify the laboratory results.

The Lister strain vaccine induced higher NE titres in primovaccines than did the Budapest strain vaccine, whereas the mouse-protective capacity of the two vaccines proved to be practically identical. On the other hand, a well-defined parallelism appeared to exist between the results obtained with the strains EM-63 and Lister in human and animal experiments. The EM-63-strain vaccine induced a weaker NE antibody response than did the Lister vaccine [9], and the former appeared to be inferior to the latter when immunized and X-irradiated mice were challenged with dermovaccinia virus [13]. A similar difference in mouse-protective capacity was shown under the present experimental conditions.

Based upon the above considerations we suppose that, though indirect laboratory methods are in any case less relevant than epidemiological observations, evaluation of the results obtained by two indirect methods may supply satisfactory information on the immunizing capacity of smallpox vaccines.

The vaccine prepared from the Lister strain proved to be superior to the Budapest strain vaccine (i) in primovaccination of children; (ii) it was at least as effective as the Budapest strain vaccine both in revaccination of humans and in the active mouse-protective test. All these results agree in that the Lister vaccine is not less immunogenic than the more reactogenic Budapest strain. It may thus be concluded that the principle that a reduction in reactogenicity would unfavourably influence the immunogenicity of smallpox vaccines is, at least in relation to the two vaccines at issue, not valid.

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ANTIBIOTICS PRODUCED BY STREPTOMYCES

IX. HEPTAFUNGIN-A, A NEW HEPTAENE MACROLIDE ANTIBIOTIC

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Summary. A new aromatic heptaene macrolide antibiotic-complex is described. It is produced by a strain defined as *Streptomyces longisporolavendulae* in a medium containing natural compounds and salts.

By countercurrent distribution, paper and thin-layer chromatographic investigation the properties of the product have been compared with other aromatic heptaenes and differences were found in the R_f value. The countercurrent distribution of the heptafungin complex yielded three peaks; the amount of the heptafungin-A fraction was 80%.

During the past years an extensive literature has accumulated on the production by *Streptomyces* sp. of polyenic macrolide antibiotics (nystatin, trichomycin, candicidin, etc.). These substances were highly active against pathogenic yeasts and yeast-like fungi but practically inactive against bacteria.

From the soil samples collected by us numerous strains producing antifungal antibiotics have been isolated. From a moist soil collection gathered at shores in Greece, a strain marked S 401 has been isolated which proved to be *Streptomyces longisporolavendulae*. The properties of the new antibiotic complex produced by this strain are discussed in the following.

Materials and methods

Media. *Streptomyces* strain S 401 was maintained on potato-glucose agar. Inoculation was carried out with spore suspension inoculated into 500 ml Erlenmeyer flasks containing 100 ml of medium of the following percentage composition: glucose, 3.0; peanut meal, 1.0; cornsteep liquor, 0.5 (50% dry weight); casein hydrolysate, 0.5 (digested by pancreatin); CaCO_3 , 0.5; NaCl, 0.3; in tap water, pH 6.8 to 7.0, before sterilization at 121 °C for 30 minutes. The medium was agitated in a rotary shaker 20 mm in diameter, at a rate of 250 r.p.m., at 28 °C for 48 hours.

Fermentation. Tank fermentations were carried out in 140 litre stainless vessels which contained 100 litres of medium of the following percentage composition: glucose, 3.0; peanut meal, 1.0; cornsteep liquor, 0.2; casein hydrolysate, 0.2; CaCO_3 , 0.5; NaCl, 0.3; NH_4NO_3 , 0.2; K_2HPO_4 , 0.1; in tap water, pH 6.8 to 7.0, before sterilization at 121 °C for 60 minutes. As antifoam, mineral oil containing 10% tributylphosphate was used.

Fermentation conditions were: temperature, 28 °C; stirring, 500 r.p.m.; air, 100 litres per minute, fermentation period, 70 to 80 hours.

The activity of heptafungin-A was assayed by the agar-diffusion method, using *Candida albicans* as test organism on the following medium: glucose, 1%; agar-agar, 2.0% dissolved in potato-juice; pH 6.8 to 7.0.

Isolation and purification. In mild acid media the antibiotic is bound to the mycelium, therefore it was filtered at pH 4.0. The filtered wet mycelium was extracted with 3 volumes of acetone at pH 7.5 to 8.0, evaporated *in vacuo* and the heptafungin complex was precipitated

by adjusting the pH to 4.5. After filtration the substance was washed in water, ether-acetone mixture, finally in petroleum ether, dried and purified by precipitation in calcium chloride-methanol solution (Chart I). The purified heptafungin complex could be fractionated by countercurrent distribution in 200 transfers, using chloroform-methanol-borate buffer (2 : 2 : 1, pH 8.4).

Paper chromatography was carried out using Whatman No. 1 filter paper in a descending system. The detection of antibiotic spots was made bioautographically using *Candida albicans*.

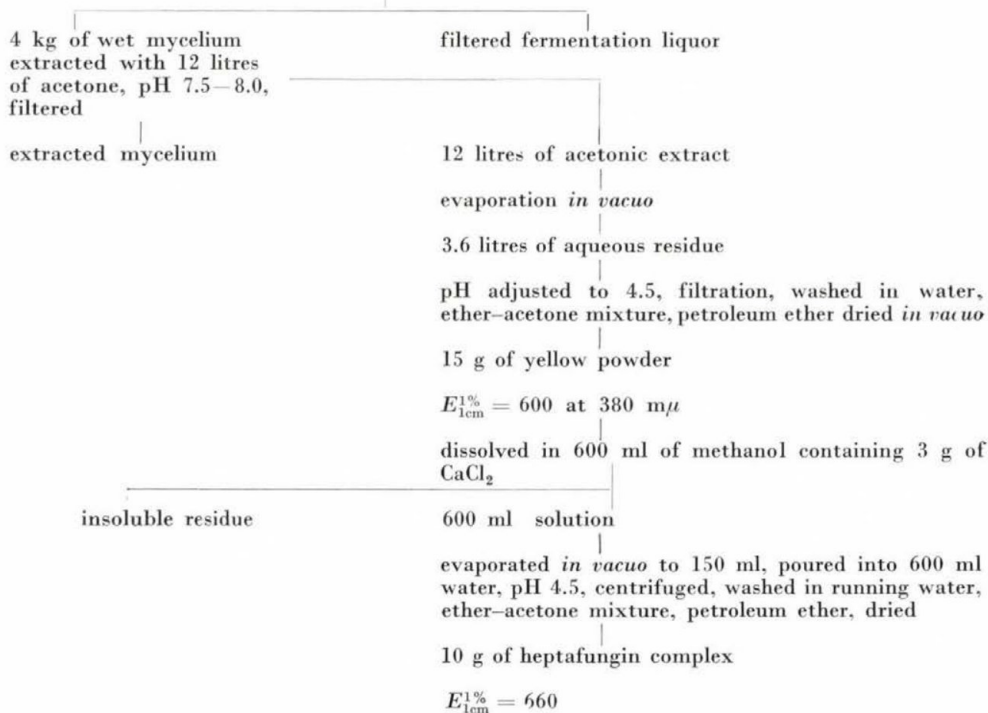
For thin-layer chromatography, 0.25 mm thin-layer Kieselgel-G was used and potassium permanganate bromophenol-blue for the detection of antibiotic spots [5].

Countercurrent distribution experiments were run in TETTAMANTI and USKERT's [7] modified countercurrent distribution apparatus. In the solvent system applied (chloroform-methanol-water; 2 : 2 : 1), the volume of the stationary (aqueous) phase (3 ml) is bound by sponges, whereas the volume of the mobile phase (chloroform) is 6 ml.

Chart I

Isolation of heptafungin complex

Fermentation broth 50 litres, biological activity 500 mcg/ml
filtered at pH 4.0



Results

Description of strain S 401. Spore chain morphology: Section Spirales. Tight spirals, usually with 5-10 turns, occur at the end of long spore chains of 10 to 70 or more spores per chain on salts-starch agar and glycerol-asparagin agar. Spore surface: smooth.

Reverse side of colony: there is no distinctive pigment (pale yellow to light yellowish brown) on yeast-malt agar, salts-starch agar or glycerol-asparagine agar.

Colour of colony: aerial mass colour in the Red colour-series ("3 ca" = pale orange yellow; "2 ca" = light ivory) on salts-starch agar and glycerol-asparagine agar.

Colour in medium: melanoid pigments are not produced in peptone-yeast-iron agar, tyrosine agar or tryptone-yeast broth. A small amount of yellow pigment may be found in the medium in oatmeal agar and salts-starch agar. The pigment is not a pH indicator.

Carbon utilization: the ability of strains to utilize various carbon compounds as sole source of carbon was determined by incorporating each carbon source separately into Pridham-Gottlieb basal salts medium and inoculating with spore suspension [2]. D-Glucose, L-arabinose, D-galactose, D-fructose, maltose, trehalose and salicin are utilized for growth. No growth or only trace of growth on rhamnose, D-xylose, lactose, melibiose, sucrose, melicitose, raffinose, inulin, adonitol, dulcitol, i-inositol and D-mannitol.

Potato-glucose agar: aerial mycelium abundant, light yellow-pink; growth wrinkled, light golden; soluble pigment yellow to yellow-brown.

Starch agar (Gause): aerial mycelium abundant, light yellow-pink; growth abundant, light yellow; no soluble pigment.

Glucose agar: aerial mycelium white, scant; growth wrinkled, cream-coloured to brown; soluble pigment light brown.

Glucose-asparagine agar: aerial mycelium well developed, yellow-pink; growth sparse, colourless to light yellow; no soluble pigment.

Glycerol-asparagine agar: aerial mycelium velvety, white turning reddish; growth thin, cream-coloured to yellowish-brown; soluble pigment light brown.

Glycerol-glycine agar: aerial mycelium at first white, then light yellow; growth cream-coloured to brownish. Soluble pigment light brown.

Glycerol-urea agar: no aerial mycelium; growth wrinkled, light brownish-yellow; soluble pigment light yellow.

Starch-casein agar: aerial mycelium velvety, light yellow; growth spreading, light yellow; no soluble pigment.

Nutrient agar: aerial mycelium absent; growth thin, light yellow; no soluble pigment.

Sucrose-nitrate agar: aerial mycelium absent or white; growth thin, colourless; no soluble pigment.

Tryptone-yeast agar: aerial mycelium thin, white, turning yellowish; growth yellowish to cream-coloured; no soluble pigment.

Yeast extract-malt extract agar: aerial mycelium velvety, white; growth abundant, yellow; no soluble pigment.

Oatmeal agar: aerial mycelium velvety, white; growth abundant, yellow; soluble pigment light yellow.

Inorganic salts-starch agar: aerial mycelium well developed, velvety, light yellowish-red; growth wrinkled, light yellow; soluble pigment yellow.

Nitrate reduction: strong.

Gelatin: no aerial mycelium; growth abundant, yellowish, spreading; liquefaction slow; no soluble pigment.

Strain S 401, which was identified on the basis of the classification system of KRASSILNIKOV [3], as a member of the Lavendulae series, shows the closest

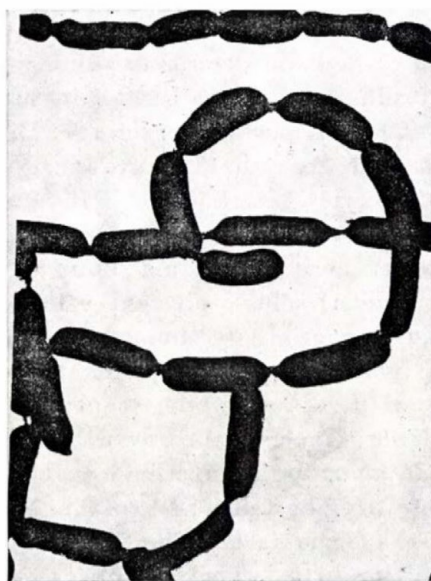


Fig. 1. *Streptomyces* strain S 401. Smooth spores; electron micrograph from 16-day-old culture on salts-starch agar

similarity with the type culture of *A. spiolavendulae* Krassilnikov (Table I). Only two species of this series of *Streptomyces*, *A. lavendofuseus* and *A. spiolavendulae* do not produce melanoid pigments [3]. *A. lavendofuseus* differs in many respects from strain S 401 and the differences which exist in microscopic morphology (Fig. 1), carbon utilization pattern and the nature of produced antibiotics between strain S 401 and *A. spiolavendulae* justify their separation at the species level. Accordingly, we consider strain S 401 to represent a new species of *Streptomyces* belonging to the Lavendulae series: *S. longisporolavendulae* n. sp. (Kalász, Széll, Gyimesi, Magyar, Horváth and Szabó, 1971); "longisporolavendulae" = long-spored, lavender colour. Type strain: S 401; deposited in CBS, Baarn.

Chemical and physico-chemical properties of the antibiotic. We subjected to further investigation fraction-A, a light yellow, amorphous powder obtained by 200 transfers of countercurrent distribution from the purified antibiotic. In methanol it shows ultraviolet maxima at 360, 380 ($E_{1\text{cm}}^{1\%} = 660$) and 402 $m\mu$. These maxima are characteristic of heptaene macrolides.

Elementary analysis: C, 61.40; H, 7.73; O, 28.52; N, 2.35%. It decomposes above 170 °C. The solubility of the antibiotic corresponds to that of other heptaene macrolide antibiotics. It is readily soluble in DMSO, DMFA, pyridine, moderately soluble in lower alcohols containing water, and in acetone, but nearly insoluble in ethyl acetate, benzene, carbon tetrachloride and petroleum ether.

The antibiotic decolorizes permanganate and bromine solutions, gives a positive Carr-Price reaction. With sulphuric acid a deep blue colour is formed. Fehling and Sakaguchi reactions are negative. Its acid hydrolysis resulted in mycosamine, and alkaline hydrolysis produced p-amino acetophenone. The method of BOROWSKI *et al.* [4] was used for hydrolysis and identification of the products.

Comparison of heptafungin-A with heptaene macrolide antibiotics of the aromatic type. Heptaene macrolides may be divided into three subgroups on the basis of N-containing products of acid and alkaline hydrolysis [5].

(1) Amphotericin B, candidin, mycoheptin, etc. belong to the non-aromatic subgroup. Mycosamine was obtained by acid hydrolysis but aromatic amine was not identified after alkaline hydrolysis.

(2) Mycosamine was found after acid hydrolysis of the antibiotics of the second (aromatic) subgroup and p-amino acetophenone was identified after alkaline hydrolysis. Candicidin, ascocin, hamycin, levorin, trichomycin, etc. belong to this group.

(3) The only antibiotic in the third subgroup is perimycin. Products of its acid and alkaline hydrolysis are perosamine and an aromatic amine, respectively.

The hydrolysis products of heptafungin are mycosamine and p-amino acetophenone, so it belongs to the second subgroup together with candicidin, trichomycin, etc. This finding excludes the identity of heptafungin and amphotericin-B (or any antibiotic of the non-aromatic subgroup). However, we wish to make a comparison between heptafungin and the members of the second subgroup.

In the course of our paper chromatographic investigations of heptaene macrolide antibiotics, the solvent system methanol-ammonia-water (20 : 1 : 4) proved to be the most suitable, in which candicidin, ascocin, hamycin and trichomycin-A behaved similarly. All of four antibiotics gave spots at $R_f = 0.00; 0.44; 0.66$. Heptafungin-A gave spots at $R_f = 0.00; 0.35; 0.55$. The results of thin-layer chromatography are demonstrated in Table II.

Table I
Comparison of characteristic properties of ten species

Species	Designation of strain	Spore chain morphology	Spore surface
<i>S. griseolavendus</i> Sumiki, 1957	ISP 5385	Section Retinaculiaperti with many straight spore chains	Smooth
<i>A. lavendofuseus</i> Krassilnikov, 1970	1973	Section Spirales; spirals with 1–2 turns	Smooth
<i>A. lavendograsseris</i> (Kouchaeva et al.) Krassilnikov, 1970	2 A 458	Section Spirales; spirals open, with 2–5 turns	Smooth
<i>A. lavendocolor</i> Kouchaeva et al., 1962	4518	Section Retinaculiaperti; hooks, loops and incomplete spirals	Smooth
<i>A. lavendosporus</i> Krassilnikov, 1970	120	Section Spirales; spirals with 2–5 turns	Smooth
<i>S. lavendulae</i> Waksman and Curtis, 1948	ISP 5069	Section Retinaculiaperti; many straight spore chains and some spirals	Smooth
<i>A. pseudolavendulae</i> (Kouchaeva et al.) Krassilnikov, 1970	3613	Section Spirales; spirals with 2–3 turns	Smooth
<i>A. spirolavendulae</i> Krassilnikov, 1970	2388	Section Spirales; short spirals with 2–3 turns	Smooth
<i>A. toxytricini</i> Gause et al., 1957	ISP 5178	Section Spirales; short spirals with 2–3 turns	Smooth
<i>S. virginiae</i> Grundy et al., 1952	ISP 5094	Section Retinaculiaperti; terminal spirals	Smooth
<i>S. longisporolavendulae</i>	S 401	Section Spirales; tight spirals with 5–10 or more turns	Smooth

We found the same paper and thin-layer chromatographic behaviour with ascosin, candicidin, hamycin and trichomycin. Since both the counter-current distribution [6, 8] and the paper chromatographic investigations revealed this close similarity, we compared only one member of the group, candicidin, to heptafungin-A, by the countercurrent distribution method (Fig. 2). In the course of these experiments candicidin and heptafungin-A solutions were charged in tube 0. After 300 transfers the respective maxima were found

of the *Lavendulae* series with strain S 401

Colour of mature aerial mycelium	Colour of substrate mycelium	Melanin production	Arabinose	Rhamnose	Xylose	Fructose	Lactose	Maltose	Sucrose	Raffinose	Inositol	Mannitol
Red	Y-b	+	—	—	—	+	N	N	—	—	—	—
Red	Y-b	—	+	+	+	+	+	—	+	—	+	+
Red	Y-b	+	—	—	—	+	—	+	—	+	N	—
Red	Y-b	+	+	—	+	—	+	+	—	—	+	—
Red	Y-b	+	+	—	+	N	—	+	—	—	—	N
Red	Y-b	+	—	—	—	+	N	N	—	—	—	—
Red	Y-b	+	—	—	+	+	+	+	—	—	+	—
Red	Y-b	—	+	—	—	+	—	+	—	+	—	—
Red	Y-b	+	—	—	—	—	+	—	—	—	—	—
Red	Y-b	+	—	—	—	+	N	N	—	—	—	—
Red	Y-b	—	+	—	—	+	—	+	—	—	—	—

Abbreviations: Y-b = yellow-brown; N = no data

in tubes 130 (heptafungin-A) and 182 (candididin):

$$K_{\text{heptafungin-A}} = \frac{130}{170} \cdot \frac{1}{2} = 0.382$$

$$K_{\text{candididin}} = \frac{182}{118} \cdot \frac{1}{2} = 0.77$$

The biological activity measurements and the distribution curves traced by the extinction at 380 m μ also revealed that this antibiotic complex consists of one major component (heptafungin-A) and of two minor ones (heptafungin-B, -C). The maxima of the respective components were found in tubes 25 (C); 70 (A) and 147 (B) (Fig. 3). On the basis of countercurrent distribution, component B was found to be identical with trichomycin-B.

Table II
Thin-layer chromatography of heptaene antibiotics

Solvent system	Amphotericin- R _f	Mycroheptin R _f	Ascocin, candidin, hamycin, levorin, trichomycin R _f	Heptafungin R _f
Butanol—ammonia—water— methanol (20 : 1 : 4 : 2)	0.07	0.06	0.13	0.10
Chloroform—methanol—borate buffer pH 8.35 (7 : 5 : 1)	0.60	0.18	0.67 0.49	0.55

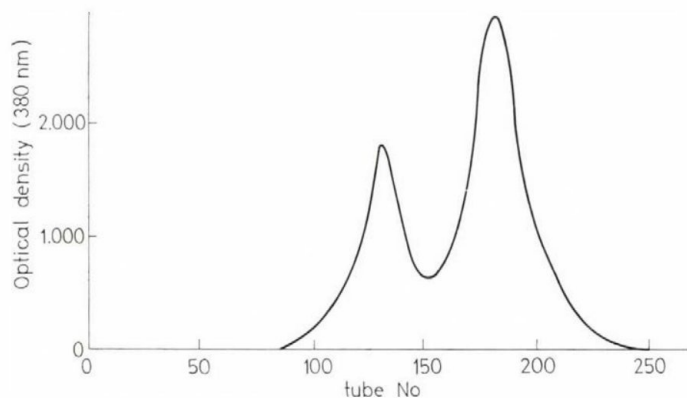


Fig. 2. Countercurrent distribution of candidin and heptafungin-A

The preparative separation of the heptafungin complex was carried out by fractionating 1.2 g of heptafungin complex by 100 transfers of countercurrent distribution with chloroform—methanol—borate buffer, pH 8.4 (2 : 2 : 1):
 Heptafungin-A fraction, 0.80 g maximum in tube 40
 Heptafungin-B fraction, 0.09 g maximum in tube 77
 Heptafungin-C fraction, 0.07 g maximum in tube 13.

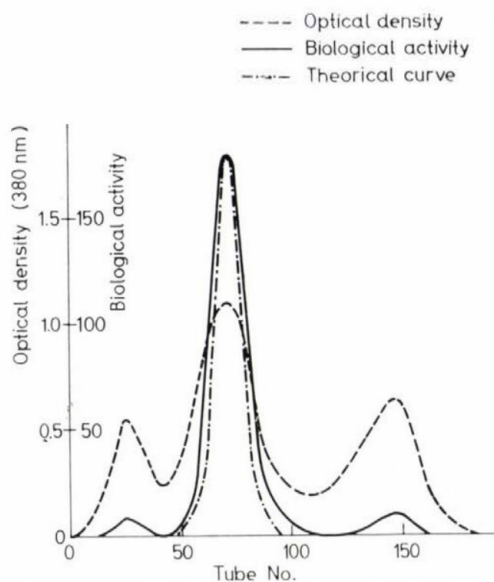


Fig. 3. Countercurrent distribution of the heptafungin complex

Table III

Microbiological spectrum of heptafungin and candidin

Strain	Minimum inhibitory concentration, $\mu\text{g/ml}$	
	Candidin	Heptafungin
<i>Fusarium moniliforme</i>	25	25
<i>Fusarium solani</i>	100	50
<i>Ophiobolus graminis</i>	12	12
<i>Trichophyton gypseum</i>	6	25
<i>Trichophyton quinckeanum</i>	3	3
<i>Microsporum canis</i>	1	6
<i>Microsporum gypseum</i>	30	12
<i>Candida albicans</i>	0.5	0.5
<i>Candida albicans</i> IAM 4888	0.5	1

Serial dilution method on Sabouraud medium.

The microbiological spectrum of the two antibiotics is practically identical.

Their intraperitoneal toxicity in suspension is also the same, 48 mg/kg.

The above findings have confirmed that heptafungin-A is a new member of the aromatic-type heptaene macrolide antibiotics.

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СТРУКТУРА И ДВИЖЕНИЕ ТРЕПОНЕМЫ

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(Поступила 15 мая 1972 г.)

Резюме. Автор показывает электронно-микроскопическую картину пучков ниточек трепонем. Он предполагает наличие связи между пучками ниточек и движением Т. Автор дает схему структуры, среза и строения ниточек и мембран трепонем.

В ходе наших электронно-микроскопических исследований мы наблюдали пучковое расположение фибрилл трепонем (Т.) На основании собственных наблюдений и наблюдений других [1—14] предположили наличие связи между ультраструктурой Т. и их движением.

Ряд авторов излагал [1—6, 10] ультраструктуру плазмы и придатков клетки. Джексон [4] вскрывает связь *envelope* и *axial filament* (а. ф.) и рисует схему структуры Т. а. ф. не является аналогичным жгутику бактерий, служащих для их движения. По структуре а. ф. можно предположить, что они играют посредническую роль между окружением вне *envelope* и телом клетки.

Правильное непрерывное штопорное движение Т. не объясняется деятельностью а. ф., идущих независимо от спиралей.

Спец. литература [1—4, 10, 11, 16] описывает ниточки или жгутики двух типов. Названия разные, но описание типов единое. На основании наблюдений других и собственных мы нарисовали схему структуры трепонемы.

Материал и метод

Трепонему получили от микробиологического отделения московского ЦКВИ Культурных Трепонем. Штамм Т—5 получен из 10-ти дневного посева. Трепонему вместе с питательной жидкостью хранили в течение 48 часов при комнатной температуре. Одну каплю осадка, полученного после хранения нанесли на часовое стекло. К поверхности капли прикосались сеткой, покрытой коллодием. Жидкость вытирали бумагой. Затем прикосались сеткой к 1% водному раствору фосфор—вольфрамовой кислоты (рН 6,8). Раствор через одну минуту отсасывали с помощью фильтровальной бумаги и препарат исследовали под советским электронным микроскопом марки УЭМБ—100. Поперечный разрез трепонемы, приготовленный по методу Овчинникова [9], из патогенных трепонем штамм (*Treponema pallidum* Nichols) показан на рис. 1-Б.

Полученные результаты

В ходе нашей работы мы приготовили такой препарат, который содержит только один тип жгутика (волокон). Путем автолиза удалили *envelope* и *axial filament*, стенку клеток и цитоплазму. Снимок (рис. №1, А, Б), приготовлен с пучков ниток под клеточной мембраной.



Рис. 1. А. Средняя часть тела Трепонема— T_{50} , содержащая только филаменты (фибриллы) ($\times 70\,000$)

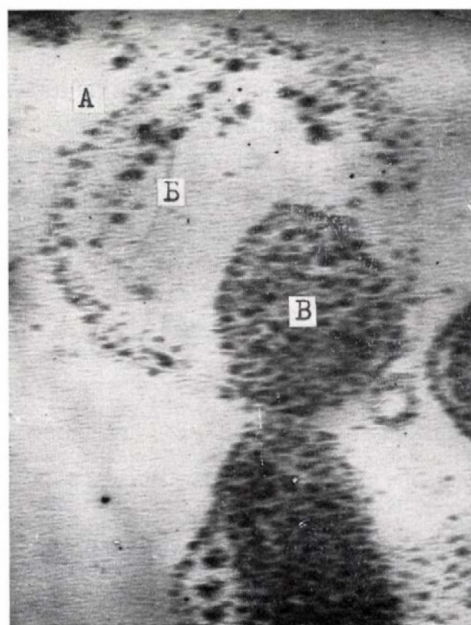


Рис. 1. Б. Тонкий поперечный разрез Т./Т (А: энвелоп; Б: аксиал-филаменты; В: цилиндрическая протоплазма) ($\times 175\,000$)

На снимке видно, что:

1. Ниточки расположены пучками (волоконнами).
2. Ход пучков спиральный.
3. Волнообразные ходы пучков фазово сдвинуты по отношению друг к другу.

Мы предполагаем, что:

1. Ритмическое повторение волокон (пучков) ниточек указывает на спиральную форму Т. (рис. №2.).

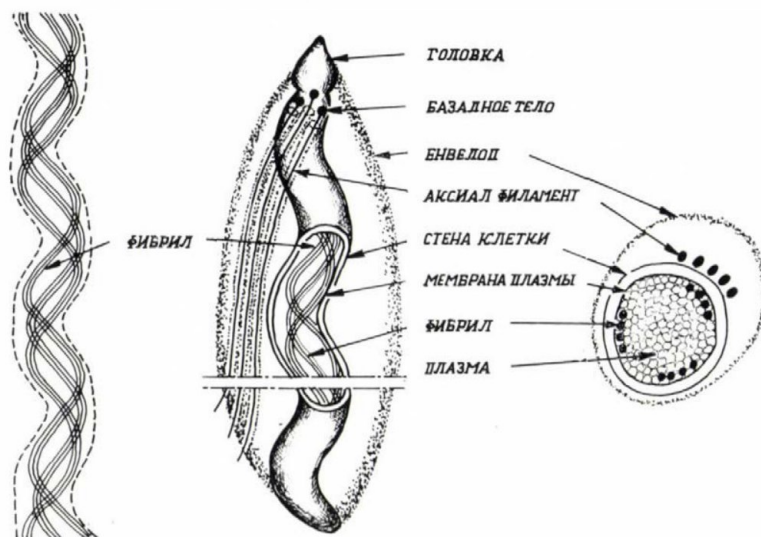


Рис. 2. Схематическое изображение Т. с аксиальными филаментами (фибриллами)

2. Волокна ниточек играют важную роль в движении Т. Такое предположение подтверждается тонкой структурой и каркасным расположением этих пучков. Типы движения Т., наблюдаемые в темном поле зрения, мы объясняем сокращением ниточек. Последовательное сокращение пучков вызывает непрерывное штопорообразное движение трепонемы. После сокращения, в фазе отдыха пучок ниточек может регенерироваться. Сокращения двух или нескольких пучков ниточек могут вызывать разгибательные и круговые движения трепонемы.

Связь между пучками ниточек и спиральной формой и схему поперечного среза и строения трепонемы мы показываем на рисунках 2/а, б, в.

Обсуждение результатов

Картину среза трепонемы не все авторы оценивают одинаково. Джексон [4] при рисунке схемы трепонемы над а. ф. показывает только энVELOП. Собственные наблюдения и наблюдения других авторов подтверждают это

понимание. Овчинников [13] публикует снимки, где кроме а. ф. видна и расширенная мембрана (рис. 2/а, б). На трепонеме, удаленной из организма вместе с зараженными тканями, должны иметься все мембраны. На срезах тканей над а. ф. фагоцитированная трепонема окружена двумя мембранами. Одна из них соответствует границе плазмы пищеварительного мешочка, другая—энвелопа Т. После фагоцитирования Т. энвелопа как будто надувается. Таким образом, полость энвелопа изолирует тело клетки Т. от окружающего ее пищеварительного мешочка.

На тех снимках, где повреждена *envelope* Т. там часто наблюдается и повреждение ультраструктуры тела клетки Т.

Снимки Джексона [3] (рис. 2, 11) подтверждают, что часто эти три мембраны (*envelope*, стенка клетки Т. и мембрана плазмы) расположены близко друг к другу. Снимки Овчинникова [10], рис. 1, 6, 14 и Овчинникова [11] рис. 26, 35, 65, 72, указывают тоже на расположение а. ф. вне двойной оболочки. На рисунке № 76/а хорошо видно, что а. ф. расположен над стенкой клетки. Автор на это и указывает. Несмотря на это, он на рисунке № 105 оболочку над «ниточками» обозначал стенкой клетки. Название «клеточная мембрана», конечно является вопросом, потому что ее свойства не совпадают со свойствами стенки клетки [15].

Простая схема трепонемы объясняется именно отличиями в названии.

Автор выражает искреннюю благодарность проф. Н. М. Овчинникову и В. В. Делекторскому за открытие возможности исследования трепонем.

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SOME CHARACTERISTICS OF THE IMMUNE RESPONSE TO CYTOMEGALOVIRUS INFECTION

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Summary. Thirty-five patients who had been subjected to open-heart surgery and had received large amounts of blood, were serially tested for antibodies to cytomegalovirus (CMV). At least seven of them gave an immune response characteristic of primary CMV infection. Another seven underwent secondary or reactivated CMV infection.

In the serum of the patients with primary infection, the antibodies demonstrable by the immunofluorescence (IF) method appeared earliest and reached the highest titre. Subsequently, the complement-fixing, and, as last, the neutralizing antibodies became demonstrable.

Among the antibodies demonstrated by the IF method, those diffusely staining the nucleus appeared earliest. These were followed by antibodies staining the perinuclear antigens and those diffusely staining the cytoplasm. The antibodies reacting with the large intranuclear granules and inclusion-like antigens as well as those reacting with cytoplasmic inclusions were the last to become demonstrable.

The antibody response to cytomegalovirus (CMV) infection has extensively been investigated by the complement-fixation (CF) [1–3] and the neutralization (NT) [1, 3, 4] tests and several reports have dealt with the antibodies demonstrable by the immunofluorescence (IF) method [2, 5].

A comparative study of the antibody responses demonstrable by the different methods as well as a detailed investigation of the antibodies reacting with antigens differentiated by the IF method may be helpful in understanding the immunological processes occurring during CMV infection and in the rapid and reliable diagnosis of such infections.

In the lack of animals susceptible to CMV, convalescent sera from patients with primary CMV infection can be used in serological tests. Such sera are obtained from a considerable part of patients who, in the course of open-heart or other surgery, have received large amounts of fresh blood.

In the present study, we have studied the dynamics of the antibody response measurable by the CF, NT and IF tests. The antibodies reacting with the different antigens localized in the infected human cells were followed up separately.

Material and methods

Immune sera. Serum samples taken serially from 35 patients who had undergone open-heart surgery.

Virus. A human CMV strain received from the virus laboratory of St. George's Hospital London, in 1964. Since then the strain has been maintained in human fibroblast cell cultures in this laboratory. Its titre fluctuated between $10^{3.5}$ and $10^{6.5}$ TCID₅₀/0.1 ml.

Cell cultures. Primary or secondary human embryonic fibroblast cultures. As medium Parker's 199 enriched with 10% bovine serum was used.

CF test. CF antigen was prepared according to ANDERSEN's method [3], the microtests were carried out in TAKÁTSY's Microtiter apparatus [6]. As CF titre the reciprocal of the serum dilution giving 25% haemolysis was taken.

Neutralization test. A virus dilution containing 200 TCID₅₀/ml was prepared in serum-free Parker No. 199 and mixed with equal amounts of 1 : 2, 1 : 10, 1 : 50 and 1 : 250 dilutions of the serum under testing. The mixtures were kept at 37 °C for one hour, then tube cultures of human embryonic fibroblasts were inoculated with 1 ml mixture each. NT titre is the reciprocal of the serum dilution giving 60% focus reduction as compared to the control focus count obtained with negative human serum.

Indirect IF test. Coverslip cultures were infected with a multiplicity of 3–5 TCID₅₀ virus/cell. After an adsorption period of 30 minutes at 37 °C, the fluid was poured off and the monolayers were washed three times with PBS and covered with maintenance fluid. The coverslips were fixed 48 hours after inoculation. The indirect IF procedure was carried out as described [7]. Anti-human rabbit gamma globulin (SEVAC, Prague) labelled with fluorescein isothiocyanate was used. In comparison with CF and NT titres, the reciprocal of the highest serum dilution which made any of the CMV antigens detectable was taken as IF antibody titre. IF antibodies to different CMV antigens were tested comparatively; the highest serum dilution which made the corresponding antigen detectable was taken into consideration.

Results

Selection of patients with primary CMV infection. Pre and postoperation serum samples obtained from 35 patients after open-heart surgery were tested by the IF method. Results are shown in Table I.

Table I

Dynamics of anti-CMV antibody response in the serum of patients with open-heart surgery

Serum samples taken								
1 to 3 days before			1 to 5 days after			25 – 60 days after		
surgery								
IF activity	Titre	No.	IF activity	Titre	No.	IF activity	Titre	No.
—	—	18	+	8—32	3	+	128—256	3
			—	—	15	+	128	4
						—	—	11
+	8—32	17	+	Not tested	17	+	128—256	7
							8—32	10

Of the 35 patients, 18 had no anti-CMV serum antibodies prior to surgery. Of these, 7 had high-titre antibodies 25 to 60 days thereafter. At the same time the remaining 11 patients had no anti-CMV antibodies. In three cases antibodies, supposedly passively introduced ones, appeared in low titres as early as 1 to 5 days after the operation. The high antibody titres (128 or 256) in the samples taken later indicated that these recipients had been infected by CMV.

In 17 cases even the pre-operation serum sample contained anti-CMV antibodies, in a titre of 8 to 32. Of these, 7 had high-titre (128 or 256) antibodies in serum samples taken 25 to 60 days after operation. In the serum of the remaining 10 patients the antibody titre had not increased by the 25th to 60th postoperative day.

Sera from patients with primary CMV infection. Serial serum samples were examined in detail from the four patients who had no IF antibodies 1 to 5 days after surgery and from one patient who had no antibodies before, but had low-titre antibody one day after operation.

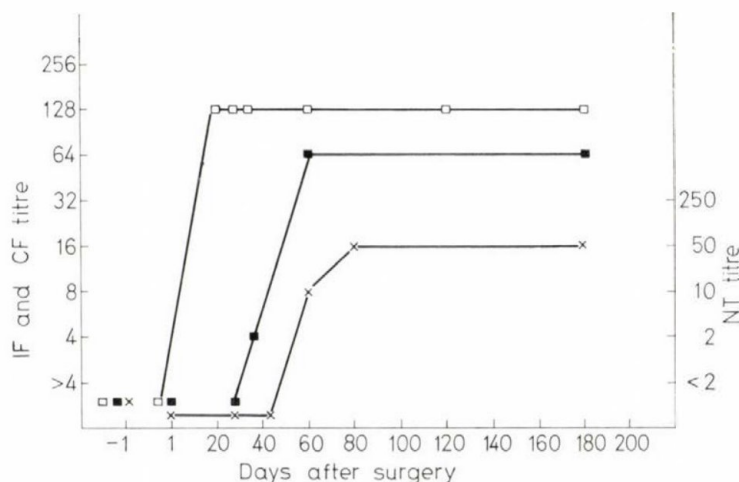


Fig. 1. Dynamics of different antibody responses to primary CMV infection (averages for four patients). Antibodies demonstrated by □—□ IF; ■—■ CF and ×—× NT tests

Fig. 1 shows the dynamics of the serum antibodies demonstrated by the IF, CF and NT methods prior to, and at different times after, surgery; the arithmetical means of titres for the four patients having no IF antibodies early after surgery have been plotted against time.

The IF antibodies had reached their highest titre (128) by the 20th postoperative day. CF antibodies appeared first around the 30th–40th day and reached their peak by the 60th day. NT antibodies were first demonstrated between the 40th and 60th day and reached their peak by the 80th day. Having reached the peak the titres showed no further change by the end of the observation period.

The antibody responses of the patient with primary CMV infection having serum antibody on the first postoperation day are shown in Fig. 2. From this patient serum was available from the 120th and 270th postoperative days as well.

On the first day after surgery, IF, CF and NT antibodies were demonstrated in low titres. The IF titre increased to 256 by the 35th day. Subsequently, it showed no further increase while the CF titre increased to 64, and the NT titre to 50, by the 120th day. On the 270th day, the IF and CF titres were tending downwards whereas the NT titre showed no change.

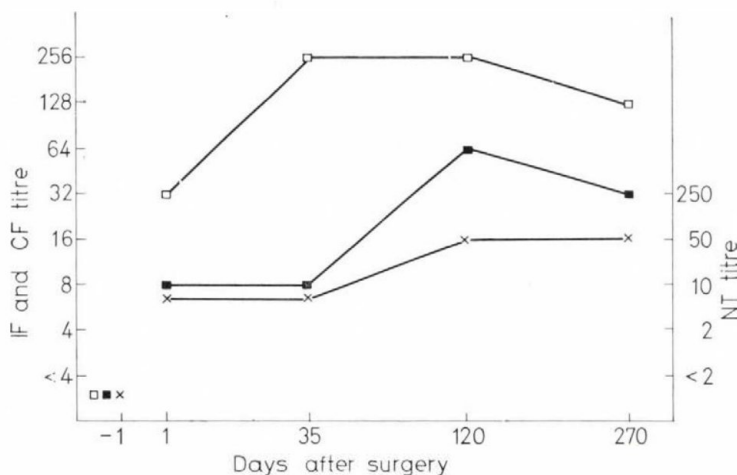


Fig. 2. Dynamics of different antibody responses to primary CMV infection (one patient).

□—□ IF; ■—■ CF and ×—× NT tests

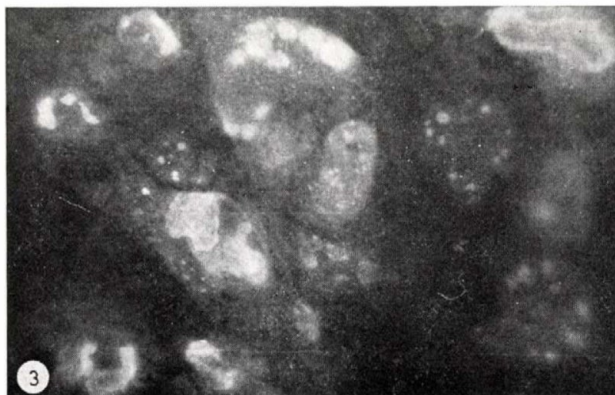


Fig. 3. Antigen(s) filling the nucleus in the form of minute granules, those forming large granules and inclusion-like bodies in the nucleus, and perinuclear antigen(s)

Localization of the antigens detected by the IF technique was examined with the 1 : 128 dilution of the serum taken from this patient on the 120th day after surgery (Figs 3 and 4).

On the basis of intracellular localization, six fluorescent antigens could be differentiated. These appeared as (i) fine granules filling the nucleus; (ii)

large nuclear granules; (iii) intracellular inclusion-like bodies; (iv) perinuclear fluorescence; (v) fluorescing substance filling the cytoplasm; and (vi) cytoplasmic inclusion-like bodies.



Fig. 4. Antigens forming large granules and inclusion-like bodies in the nucleus, perinuclear antigens, those responsible for diffuse cytoplasmic fluorescence and the antigens forming inclusion-like bodies in the cytoplasm

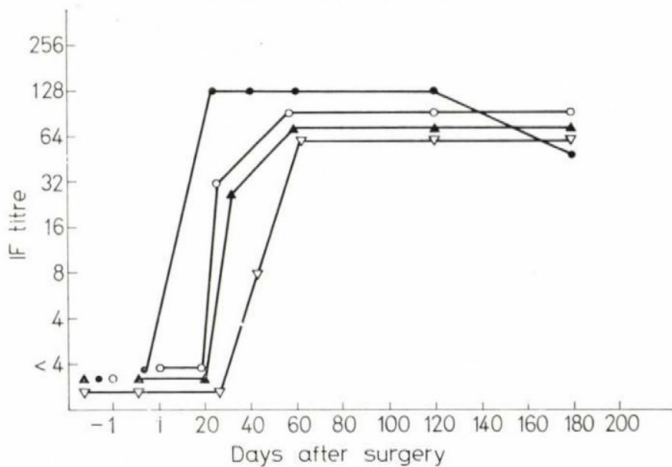


Fig. 5. Dynamics of antibody responses to antigens of different location in the course of CMV infections. Antibodies reacting with ●—● diffuse nuclear; ○—○ perinuclear; ▲—▲ diffuse cytoplasmic antigens and ▽—▽ antigens forming large nuclear granules; nuclear inclusion-like bodies and cytoplasmic inclusion-like bodies

Dynamics of the antibody response to different antigens. Fig. 5 shows the average antibody response of the four patients who had no antibodies immediately after surgery. The arithmetical means of the antibody titres have been plotted against time.

It is clearly seen that the antibodies appearing in the first 20-day period following surgery were those directed against the antigens marked (i) in the

above list. Antibodies to antigens (iv) and (v) appeared in the period from the 20th to the 40th postoperative day, while antibodies to antigens (ii), (iii) and (vi) were first demonstrated between the 30th and 60th days. The titres showed no change between days 60 and 120. By the 180th day, the antibodies to the diffuse intranuclear antigen(s) had shown some decline, the other antibodies had not changed in titre.

Discussion

It has been found that open-heart surgery and transfusion of large amounts of fresh blood were accompanied by primary CMV infection in 7 cases, by secondary or reactivated CMV infection in another 7 cases, among the 35 cases tested. Since it may occur that anti-CMV antibodies are first detected after the 60th day, the negative result in the remaining 21 cases cannot be accepted with certainty.

Primary CMV infection has been observed after transfusion of large amounts of fresh blood [8–12] in such a high percentage [11, 12] which cannot exclusively be explained by virionaemia of the donors. It must be assumed that donors who have undergone CMV infection and are serologically positive, may carry the virus in a nonproductive state and consequently serve as potential sources of CMV infection. CMV has been isolated from peripheral leucocytes or selected lymphocytes of several patients with the post-transfusion syndrome [13–16]. Thus, it seems that the virus is carried by leucocytes or lymphocytes. According to DRÓSI *et al.* [17] and PERHAM *et al.* [12], 10 to 12% of the donors may be a source of CMV infection.

In 17 patients we have found antibodies to CMV before surgery and 7 of the 17 patients gave after the operation an antibody response. In these cases latent infections might have been re-activated or, else, the transfusion resulted in a new infection with an antigenically distinct [18], or identical, CMV strain.

In the 14 cases observed by us the infection was subclinical. However, CMV infection may manifest itself with severe illness such as infectious mononucleosis, hepatitis, pneumonitis, etc., [1, 19, 20].

The antibodies detectable by the IF test were the first to appear in the course of infection. The CF antibodies appeared later and attained lower titres, and the NT antibodies had become demonstrable still later. The titres persisted for long periods of time at the same level. In the case illustrated in Fig. 2, the NT titre had not begun to decline even by the 270th day. By the same time, the IF and CF titres had decreased by one dilution step.

The low NT titres in the present study might be attributed to the fact that we did not use complement in this test. ANDERSEN [4] showed that the neutralizing capacity of antibodies, especially of those of the IgM class, is enhanced by complement in relation to several types of cytomegalovirus. It

may also be supposed that the anti-CMV neutralizing antibodies are strain-specific, *i.e.*, give low titres with heterologous strains [3].

Based on their localization and morphology in CMV-infected human embryonic fibroblasts, we have been able to distinguish six different antigens. The serum antibodies reacting with these antigens appeared at different times in the course of the antibody response. The antibodies responsible for diffuse nuclear fluorescence appeared earliest and reached the highest titres. Those reacting with the perinuclear and diffuse cytoplasmic antigens were also characteristic of the early immune response. The antibodies giving fluorescence with large nuclear granules and nuclear or cytoplasmic inclusion-like bodies were either absent in the early sera or their titre was low. In late serum samples, these antibodies reached titres as high as those of early antibodies.

TOKUMARU [21] and GÉDER *et al.* [22] demonstrated an increase in the IgM-type antibodies characteristic of the early phase of the immune response to herpes simplex virus infection. In the course of the CMV post-transfusion syndrome the IgM-type serum antibodies appeared earlier and reached higher titres than did the CF antibodies [16]. CHIANG *et al.* [23] investigating human CMV infection, demonstrated the highest IgM-type antibody titres in sera taken in the early phase of the illness.

On the basis of these data, we suppose that the antibodies which reacted first with the diffuse nuclear antigens and those reacting with the perinuclear and diffuse cytoplasmic antigens mainly belonged to the IgM class. This is consistent with observations published by HANSHAW *et al.* [5], but inconsistent with the findings of SCHMITZ and HAAS [24]. The latter authors succeeded in detecting the large intranuclear granules and the inclusion-like bodies by separated IgM globulins.

The dynamics of the antibody response to the large nuclear granules and cytoplasmic inclusion-like antigens showed some parallelism with the dynamics of the CF antibody response. Similar findings were reported by JACK and WARK [25], who failed to detect CF antigen in a serum which did not react with large nuclear granules and inclusions.

Since the appearance and the high titre of IgM antibodies are thought to be characteristic of a primary CMV infection [5, 16, 23] and such antibodies are often demonstrable in the absence of measurable amounts of CF and NT antibodies (Figs 1 and 2), tests for the demonstration of IgM-type antibodies seem to be useful in the early diagnosis of CMV infections. The diagnosis would be made even more reliable by demonstrating whether the macroglobulins induced by different CMV strains are homogeneous serologically.

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THE EFFECT OF METHICILLIN IN COMBINATION WITH TRYPSIN AND LYSOZYME ON THE GROWTH OF METHICILLIN SENSITIVE AND RESISTANT STAPHYLOCOCCUS AUREUS STRAINS

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Summary. Using a standard tube dilution assay, in the presence of methicillin the combination of trypsin (100 $\mu\text{g/ml}$) and lysozyme (500 $\mu\text{g/ml}$) rendered both methicillin-sensitive and adaptively resistant strains of *Staphylococcus aureus* significantly more susceptible to methicillin. In contrast, the degree of methicillin resistance in a highly methicillin-resistant mutant of a naturally occurring methicillin-resistant isolate of *Staph. aureus* 5814/1966 was significantly increased in the presence of the two enzymes.

In biophotometric studies, the growth of methicillin-sensitive and adaptively resistant strains was retarded, whereas the multiplication of the highly methicillin-resistant mutant was promoted by the combination of the two enzymes in the presence of methicillin.

When late in the logarithmic phase a subinhibitory concentration (1 $\mu\text{g/ml}$) of methicillin and the two enzymes were added to the culture of the methicillin sensitive strain 100, which had previously been grown in the absence of these substances, the number of surviving cells was decreased by 6 exponents. The addition of methicillin resulted in a 5-exponent decrease. In contrast, the addition of a subinhibitory dose (800 $\mu\text{g/ml}$) of methicillin to the late logarithmic phase culture of the highly methicillin-resistant mutant failed to influence the viable count. On the addition of this concentration of methicillin simultaneously with the two enzymes, the decrease amounted to 1 exponent.

MARTIN and SWORD reported that methicillin-sensitive strains of *Staph. aureus* lose their lysozyme resistance in the presence of methicillin [11] while WARREN and GRAY found that treatment with methicillin renders the normally lysozyme-resistant cells of *Staph. aureus* susceptible to partial lysis by lysozyme and the concomitant addition of trypsin resulted in a further dissolution of the cells [25]. On the other hand, we have recently shown that the highly methicillin-resistant mutants of naturally occurring methicillin-resistant isolates of *Staph. aureus* are able to maintain their resistance to both lysozyme and trypsin in the presence of methicillin at subinhibitory concentrations. On the basis of these findings it has been concluded that naturally methicillin-resistant staphylococci may differ from methicillin-sensitive ones in cell wall synthesis and composition [14, 19].

The purpose of this paper is to present experiments in which the interaction of methicillin, trypsin and lysozyme on the growth of *Staph. aureus* strains sensitive and resistant to methicillin has been studied under various conditions.

Materials and methods

The materials and methods were for the most part described previously [19]. The essential information is summarized as follows. Three types of experiments were carried out with methicillin-sensitive, adaptively methicillin-resistant and naturally resistant strains of *Staph. aureus*.

(1) The minimum inhibitory methicillin concentration in the presence of the two enzymes was determined by using a standard tube dilution assay with a final inoculum of 10^6 organisms/ml.

(2) For studying the multiplication of the strains in the presence of methicillin and of methicillin + enzymes, the Bonet Maury et Jouan Biophotomètre was used.

(3) The effects of methicillin and of methicillin + enzymes on late logarithmic phase cultures, which had previously been grown in the absence of these substances, were examined also in the biophotometer, and the viable cells were determined after 22–24 hours incubation by the plate count method. All incubations were carried out at 37 °C.

Statistical evaluations were performed in the Computer Centre of Kossuth Lajos University, Debrecen.

Results

Changes in methicillin susceptibility in the presence of trypsin and lysozyme. Before investigating the common effect of trypsin and lysozyme on the degree of methicillin resistance in *Staph. aureus* strains of different methicillin sensitivity, the growth of strains 100 and 5814R in the presence of trypsin (100 µg/ml) + lysozyme (500 µg/ml) was examined in the biophotometer. The combination of the two enzymes failed to influence the shape of growth curves either for the methicillin-sensitive or for the naturally methicillin-resistant strain.

Table I

Effect of trypsin (100 µg/ml) and lysozyme (500 µg/ml) on susceptibility to methicillin of Staph. aureus strains of different methicillin sensitivity

Media	Incubation, hours	Minimum inhibitory concentration for methicillin,* µg/ml			
		methicillin sensitive		methicillin resistant	
		<i>Staph. aureus</i> 100	<i>Staph. aureus</i> 80/81	<i>Staph. aureus</i> 18R adaptive	<i>Staph. aureus</i> 5814R natural
Control	24	1.5 ± 0.58	2 ± 0	37.3 ± 8.3	1200 ± 0
Enzymes		0.38 ± 0.14	0.31 ± 0.13	20.0 ± 4.6	1600 ± 0
		P = 0.05	P < 0.001	P < 0.01	P < 0.001
Control	48	1.5 ± 0.58	4 ± 0	64 ± 0	1600 ± 0
Enzymes		0.63 ± 0.25	0.63 ± 0.25	28 ± 4.6	2400 ± 0
		P = 0.05	P < 0.001	P < 0.001	P < 0.001

* Average values for 4 experiments.

The data in Table I show that the combination of the two enzymes in the presence of methicillin rendered both methicillin-sensitive and adaptively resistant strains significantly more susceptible to methicillin. In contrast, the degree of methicillin resistance in the naturally methicillin-resistant mutant was significantly elevated by the two enzymes.

Influence of enzymes on the dynamics of growth in the presence of methicillin. Since the standard tube dilution assay can reveal no detail of the nature of multiplication of cells under various conditions, the effect of enzymes in the presence of methicillin on the growth of *Staph. aureus* strains of different methicillin sensitivity was examined in the biophotometer.

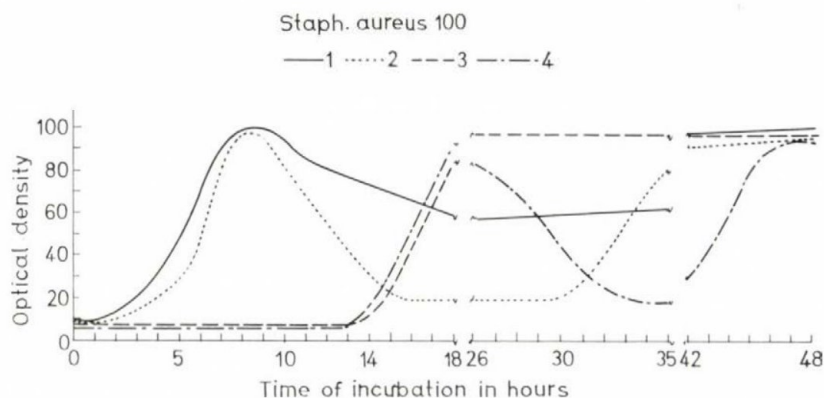


Fig. 1. Growth curves for penicillinase-negative, methicillin-sensitive *Staph. aureus* strain 100 in Difco-casitone broth. 1 = methicillin, 0.5 $\mu\text{g/ml}$; 2 = methicillin, 0.5 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$; 3 = methicillin, 0.5 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$; 4 = methicillin, 0.5 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$

The interaction of trypsin + lysozyme + methicillin on the growth of the methicillin-sensitive strain 100 can be seen in Fig. 1. For comparison, growth curves recorded in the presence of methicillin, trypsin + methicillin, and of lysozyme + methicillin are presented.

It can be seen that in the presence of methicillin + trypsin + lysozyme the lag phase was lengthened (curve 4) to such an extent as was observed with methicillin + lysozyme. The logarithmic phase was similar to those seen in any other medium. After an about 8-hour stationary phase the optical density fell rapidly and reached the lowest level in 8 hours. This optical density remained constant for about 5 hours. Further incubation resulted in a secondary exponential phase. No methicillin inactivation was found.

The growth dynamics for methicillin-sensitive and adaptively resistant staphylococcal strains were similar in the presence of methicillin, and of methicillin + enzymes.

The interaction of methicillin and enzymes on methicillin-sensitive cells at various stages of growth may be composed of the separate effects of methicillin + lysozyme, and of methicillin + trypsin. The prolonged lag phase may be due exclusively to the interplay of methicillin + lysozyme. Curves 3 and 4 suggest that in methicillin-sensitive cultures lysozyme acts primarily on lag phase cells if cultivation occurs in the presence of methicillin from the very beginning of incubation. Since methicillin similarly to penicillin [6, 12, 16, 24, 26, 27] is supposed to inhibit the final or cross-linking step in peptidoglycan synthesis of *Staph. aureus*, part of the inoculated cells may undergo lysis by methicillin. However, most cells, although possessing non-cross-linked peptidoglycan, can multiply in the presence of methicillin. In contrast, if lysozyme is present in the medium, it destroys all the cells producing defective peptidoglycan in which the β -1,4-glycosidic linkages are available. Hence, only a small number of cells, in which peptidoglycan synthesis is not affected by methicillin, tolerates the lytic action of lysozyme and will be able to multiply. The daughters of these cells seem, however, to have peptide bonds vulnerable by trypsin.

The large fall in the optical density of the stationary phase culture in the presence of methicillin + trypsin + lysozyme may be due exclusively to the interaction of methicillin + trypsin. As curves 2 and 4 indicate, trypsin seems to act primarily on stationary phase cells if cultivation occurs in the presence of methicillin from the very beginning of incubation. In such a case, the particular peptide bonds [10] in the peptide portion of peptidoglycan, the synthesis of which is partially inhibited by methicillin, allow easy access for trypsin, and the loss of integrity of peptidoglycan and the concomitant cell lysis are accelerated by the hydrolytic action of trypsin.

The secondary logarithmic phase does not seem to be a real exponential phase of slightly damaged methicillin-sensitive cells capable of surviving the common effect of methicillin + lysozyme + trypsin. It rather seems to represent the first logarithmic phase of the methicillin-resistant minority of the population, which, as demonstrated previously [18], shows a long lag phase.

Fig. 2 illustrates the action of methicillin + trypsin + lysozyme (curve 4) on the growth of the highly methicillin-resistant mutant derived from the naturally occurring methicillin-resistant strain 5814/1966 of mixed population. For comparison, growth curves obtained in the presence of methicillin, trypsin + methicillin, and of lysozyme + methicillin are shown.

It can be seen that in the presence of methicillin + trypsin + lysozyme the lag phase was similar to that obtained in methicillin + lysozyme and was half as long as those observed either in methicillin or in methicillin + trypsin. The logarithmic phase and the early stationary phase were similar in all media. Late in the stationary phase, the optical density was slightly decreased by methicillin + enzymes.

The promoted lag phase of methicillin-resistant culture in the presence of methicillin + trypsin + lysozyme may be attributed exclusively to the interplay of methicillin + lysozyme. Two processes are assumed to take place. First, the methicillin-sensitive minority of the population is lysed. Second, in the resistant cells lysozyme may help to bring about an appropriate balance between glycan-synthetizing and hydrolyzing reactions resulting in thinner cell walls and a shorter generation time. This hypothesis is based on our recent finding of methicillin-resistant cells having much thicker walls than their methicillin-sensitive counterparts (See Discussion).

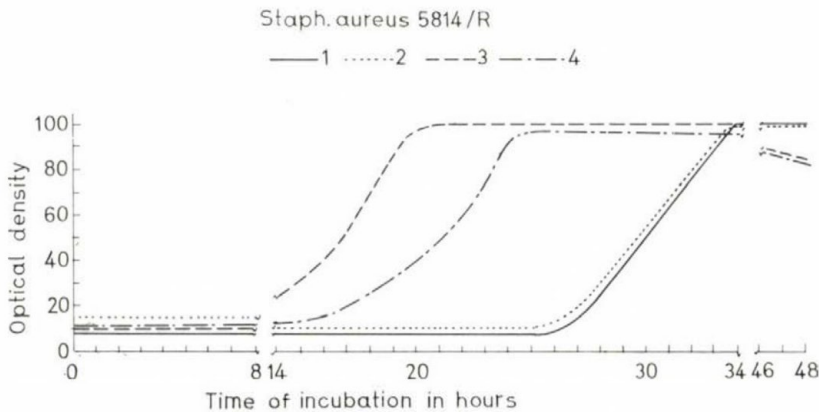


Fig. 2. Growth curves for penicillinase-producing, naturally methicillin-resistant *Staph. aureus* strain 5814R in Difco-casitone broth. 1 = methicillin, 800 $\mu\text{g/ml}$; 2 = methicillin, 800 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$; 3 = methicillin, 800 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$; 4 = methicillin, 800 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$

The moderate lysis late in the stationary phase may be the result of the destruction of those old cells in which the natural disintegration of walls is accelerated by the hydrolytic action of the two enzymes.

Effect of methicillin + enzymes on late logarithmic phase cultures. Since the results presented were suggesting that the response of methicillin-sensitive cells to the interaction of methicillin + enzymes differed from that of the highly methicillin-resistant mutant, it was considered of interest to study the behaviour of cultures of different methicillin sensitivity after adding methicillin + enzymes to them late in the logarithmic phase. The strains were cultured in the biophotometer in broth containing neither enzymes nor methicillin. Late in the logarithmic phase, enzymes, methicillin, and enzymes + methicillin were added to different cuvettes and the further course of growth was recorded. The viable count of each culture was determined after 24 hr incubation.

Control experiments showed that the combination of the two enzymes had no significant effect on survival of the cells of methicillin-sensitive strains

100 and 80/81, and of the naturally methicillin-resistant mutant 5814R. However, a significant reduction in viable count of the adaptively resistant strain 18R was found. For this reason, strain 18R was omitted from the further experiments.

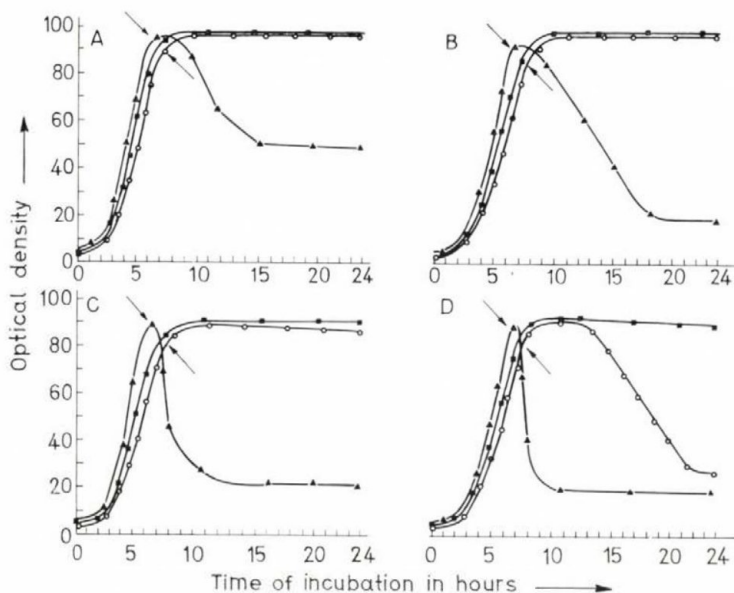


Fig. 3. Effect of methicillin, and of methicillin + enzyme(s) on optical density of logarithmic phase cultures sensitive and resistant to methicillin. Arrows indicate the addition of various substances. Section A: lysis of *Staph. aureus* strain 100 after adding subinhibitory concentration (1 $\mu\text{g/ml}$) of methicillin $\blacktriangle$ - $\blacktriangle$; lysis of *Staph. aureus* strain 5814R after adding subinhibitory concentration (800 $\mu\text{g/ml}$) of methicillin $\blacksquare$ - $\blacksquare$, and inhibitory concentration (1200 $\mu\text{g/ml}$) of antibiotic $\bullet$ - $\bullet$. Section B: lysis of *Staph. aureus* strain 100 after adding methicillin, 1 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$ $\blacktriangle$ - $\blacktriangle$; lysis of *Staph. aureus* strain 5814R after adding methicillin, 800 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$ $\blacksquare$ - $\blacksquare$; and methicillin, 1200 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$ $\bullet$ - $\bullet$. Section C: lysis of *Staph. aureus* strain 100 after adding methicillin, 1 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$ $\blacktriangle$ - $\blacktriangle$; lysis of *Staph. aureus* strain 5814R after adding methicillin, 800 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$ $\blacksquare$ - $\blacksquare$; and methicillin, 1200 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$ $\bullet$ - $\bullet$. Section D: lysis of *Staph. aureus* strain 100 after adding methicillin, 1 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$ $\blacktriangle$ - $\blacktriangle$. Lysis of *Staph. aureus* strain 5814R after adding methicillin, 800 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$ $\blacksquare$ - $\blacksquare$; and methicillin, 1200 $\mu\text{g/ml}$ + trypsin, 100 $\mu\text{g/ml}$ + lysozyme, 500 $\mu\text{g/ml}$ $\bullet$ - $\bullet$.

The effect of methicillin + enzymes on the optical density of late logarithmic phase cultures of methicillin-sensitive strain 100 and methicillin-resistant mutant 5814R is illustrated in section D of Fig. 3. For comparison, the effect of methicillin, methicillin + trypsin and of methicillin + lysozyme is shown in sections A, B and C.

The curves illustrated in section D show that the simultaneous addition of the two enzymes and of a subinhibitory dose (1 $\mu\text{g/ml}$) of methicillin to the sensitive culture of strain 100 caused the most rapid and largest fall in optical

density. In contrast, the optical density of the resistant culture of mutant 5814R was only slightly decreased by methicillin at the subinhibitory concentration of 800 $\mu\text{g/ml}$ in the presence of the two enzymes. However, adding to the resistant culture an inhibitory dose (1200 $\mu\text{g/ml}$) of methicillin with the two enzymes rendered part of the cells susceptible to the lytic action of the antibiotic-enzyme combination, and a considerable fall in optical density could be observed.

In order to study the quantitative results of the lytic effect of the combination of methicillin and enzymes on the viable count of cultures with different methicillin sensitivity, in each culture the number of colony-forming units was determined in the 22nd–24th hour.

Table II

Effect of enzymes, methicillin and of enzymes + methicillin on survival of logarithmic phase cultures of Staph. aureus strains

Substances added to logarithmic phase cultures	Number of viable cells* per ml		
	<i>Staph. aureus</i> 100 (1)	<i>Staph. aureus</i> 5814 R	
		(800)	(1200)
Broth	$1.6 \pm 0.3 \times 10^9$	$1.0 \pm 0.1 \times 10^9$	$1.1 \pm 0.1 \times 10^9$
100 μg trypsin/ml + 500 μg lysozyme/ml	$1.9 \pm 1.0 \times 10^9$ $P < 0.6$	$1.1 \pm 0.2 \times 10^9$ $P > 0.9$	$1.1 \pm 0.2 \times 10^9$ $P > 0.9$
Methicillin	$2.6 \pm 0.5 \times 10^4$ $P = 0.001$	$1.2 \pm 0.7 \times 10^9$ $P = 0.5$	$6.6 \pm 2.4 \times 10^8$ $P = 0.01$
100 μg trypsin/ml + 500 μg lysozyme/ml + methicillin	$7.1 \pm 0.7 \times 10^3$ $P < 0.001$ $P_1 = 0.001$	$1.1 \pm 0.1 \times 10^8$ $P < 0.001$ $P_1 = 0.01$	$1.3 \pm 1.0 \times 10^6$ $P < 0.001$ $P_1 = 0.001$

* Means were calculated from the viable counts on 5 plates. Figures in brackets indicate the concentrations of methicillin, $\mu\text{g/ml}$, added to logarithmic phase cultures. P_1 was calculated for mean found in methicillin.

The data presented in Table II indicate that a subinhibitory concentration of methicillin significantly reduced the viable count in the sensitive culture, whereas there was no drop in the resistant culture. Of course, an inhibitory amount of the antibiotic resulted in a significant drop in viability of the resistant culture. However, the decrease was numerically much less than that produced by a subinhibitory dose of methicillin in the sensitive culture.

The effect of the two enzymes and methicillin resulted in a significant decrease in the number of viable cells in each culture. Nevertheless, after

adding a subinhibitory concentration of methicillin with the two enzymes to the sensitive culture, the viable count was reduced by 6 exponents, whereas in the resistant culture the number of surviving cells was decreased by 1 exponent only. Even in the presence of the two enzymes, an inhibitory amount of the antibiotic was needed for the resistant culture to produce as large a drop in viability as that induced by a subinhibitory dose in the sensitive culture.

Finally, the quantitative effect of the combination of enzymes with methicillin at different concentrations on the number of surviving cells was also calculated in relation to that of methicillin. The decreasing effect of the enzyme-antibiotic combinations was found to be significant in each culture. On the other hand, the results presented indicated a considerable difference between sensitive and resistant cells. In the sensitive culture, most of the damaged cells were lysed by methicillin. In contrast, in the resistant culture most of the injured cells needed for lysis the additional effect of the two enzymes.

Discussion

The precise mechanism of methicillin resistance in *Staph. aureus* is unknown, although a number of authors have investigated the problem [2-5, 7-9, 14, 18-23]. The component of the staphylococcal cell wall responsible for the shape and rigidity of the cell is peptidoglycan, a complex polymer [12]. Our previous [14, 19] studies and the present one were based on the assumption that the cell wall synthesis in methicillin-resistant staphylococcal cells was insensitive to the inhibitory effect of methicillin at concentrations allowing growth. The present results have confirmed this assumption. The fact that the addition of a subinhibitory dose (800 $\mu\text{g/ml}$) of methicillin to exponentially growing cells of the methicillin-resistant strain fails to decrease the viable count, and the simultaneous administration of trypsin and lysozyme results in a reduction by 1 exponent in the number of surviving cells, indicates that methicillin at even such a high concentration is unable to inhibit in most part of the cells the enzymic reactions responsible for normal wall synthesis. Of course, when the antibiotic is applied at a concentration of 1200 $\mu\text{g/ml}$, one sufficient for stopping the normal synthesis of peptidoglycan in a number of cells, the lysis of these particular cells by the combination of trypsin and lysozyme will be demonstrable.

One of the ways in which cells could be resistant to methicillin would be to have a higher proportion of their cell mass as cell wall material, so that the inhibition of peptidoglycan synthesis would have no or only a weak effect on the viability of the cells. DYKE [5] has found no greater proportion of cell wall material either in a heterogeneous resistant strain or its highly resistant homogeneous mutant than in either the Oxford *Staphylococcus* strain or the penicillinase-negative mutant No. 524SC(N). On the other hand, PARK *et al.* [13]

have recently reported highly penicillin-resistant mutants of *Staph. aureus* H to differ from the parent in containing significantly more murein per milligram of cells. We have studied the differences in fine structure between a methicillin-sensitive and a resistant mutant of *Staph. aureus* 5814, and the sensitive mutant appeared to have a thinner cell wall than its methicillin-resistant counterpart (see Figs 4 and 5).

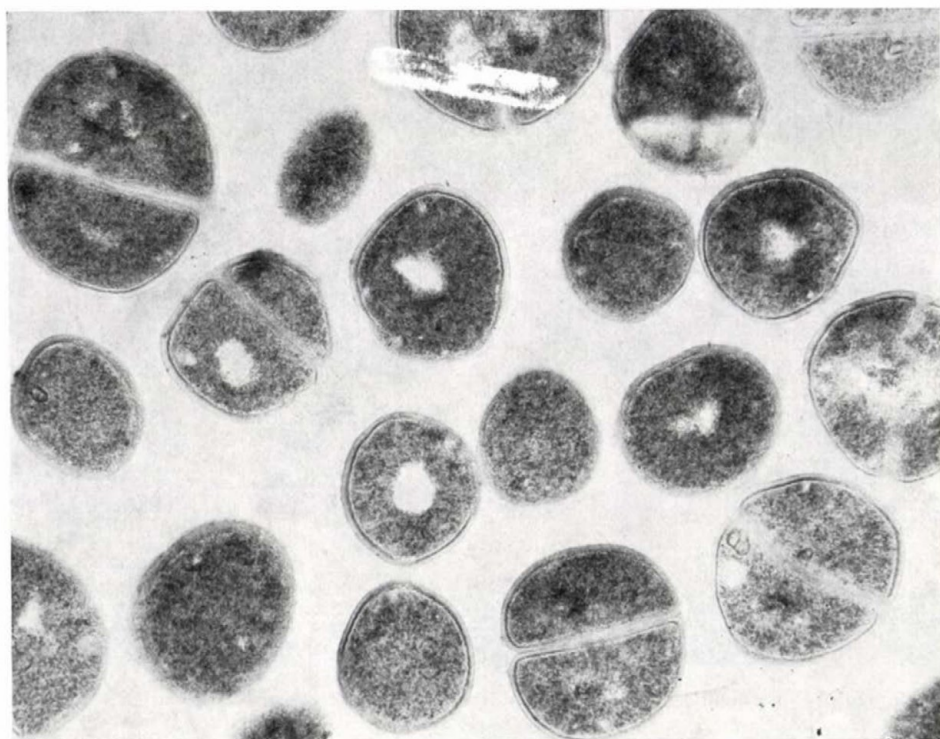


Fig. 4. Methicillin-sensitive cells derived from heterogeneous resistant strain 5814 ($\times 30\,800$)

Although the alteration in cell wall synthesis seems to be of decisive importance in respect of methicillin resistance, the methicillin-resistant cells appear to differ from the sensitive ones also in some membrane properties. We have recently shown that the degree of methicillin resistance in highly methicillin-resistant mutants is significantly decreased by 1 M NaCl [20]. We have also found the composition of phospholipids in methicillin-resistant cells to be different from that in methicillin-sensitive cells [21].

In Gram-negative bacteria there seems to be some relationship between membrane-bound phosphatidyl ethanolamine and certain wall synthesizing enzymes [17]. In *Staph. aureus* phospholipid carriers play also some role in the

synthesis of peptidoglycan [1]. The nature of these phospholipids has not been clarified. On the basis of our recent indirect findings, lysyl-phosphatidyl glycerol is supposed to be one of the essential functioning lipids in staphylococcal cell membranes [15, 21].

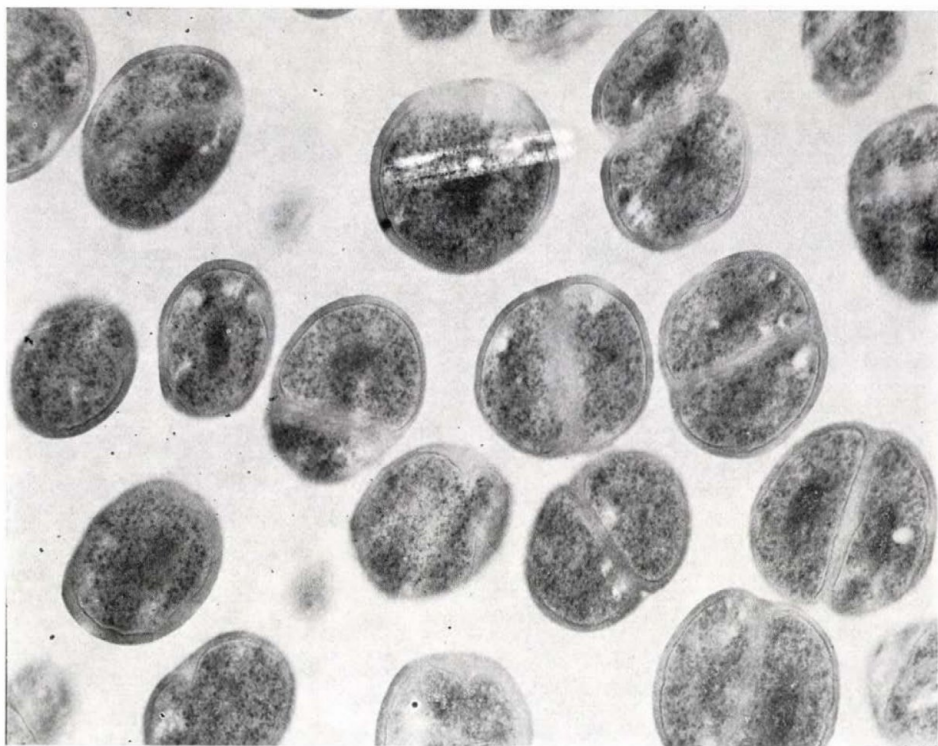


Fig. 5. Highly methicillin-resistant cells derived from heterogeneous resistant strain 5814 ($\times 30\ 800$)

Finally, it is assumed that one of the reasons why staphylococcal cells can be resistant to methicillin may be the increased insensibility of the wall synthesizing enzyme(s) against methicillin.

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MICRONUTRIENT STUDIES ON SOME FUNGI CAUSING LEAF SPOT DISEASE

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Summary. The effects of zinc, iron, copper and manganese on growth and sporulation of *Alternaria tenuis* (Auct.), *Colletotrichum capsici* (Syd.) Butler and Bisby and *C. gloeosporioides* (Penzig) isolated from the diseased leaves of *Lycopersicon esculentum* (tomato), *Scindapsus pictus* and *Polyscias balfuriana*, respectively, have been studied. All the four trace elements were found to be essential for vegetative growth and sporulation. The amounts of copper and manganese required by these organisms for maximum growth and sporulation were comparatively smaller than those of iron and zinc. The optimum concentrations of trace elements for growth and sporulation of the present organisms have been determined.

Trace metals play an important role in the nutrition of fungi. As constituents or activators of enzyme systems they catalyze vital reactions in the pathogens. The essentiality of micro elements was recognized following the classical work of STEINBERG [20] who showed the necessity of trace elements for growth and sporulation of *Aspergillus niger*. Subsequent studies on the indispensability of trace elements for growth of fungi have been established by several workers (FOSTER [6], LILLY and BARNETT [10], PERLMAN [13], SARASWATHI-DEVI, [18], DAFTARI [4], SANKHLA and MATHUR [15], THIND and RAWLA [25], SINGH and PRASAD [19], THIND and MANDAHAR [24], BHATNAGAR and PRASAD [3], RAWLA [14], APPARAO [2], SANKHLA *et al.* [16], MANDAHAR [11]. In the present paper, the effect of the four trace elements zinc, manganese, copper and iron has been studied on the growth and sporulation of three fungi causing leaf spot disease.

Material and methods

Alternaria tenuis (Auct.), *Colletotrichum capsici* (Syd.) Butler and Bisby, and *C. gloeosporioides* (Penzig) included in the present investigation were isolated from the diseased leaves of *Lycopersicon esculentum* (tomato), *Scindapsus pictus* and *Polyscias balfuriana*, respectively. Glassware was cleaned in a sulphuric acid—potassium dichromate solution and then well rinsed with tap water and finally with doubly distilled water. The culture medium contained glucose, 5.0 g; KNO₃, 3.5 g; KH₂PO₄, 1.75 g; MgSO₄ · 7H₂O, 0.75 g; and 1000 ml doubly distilled water. This medium was purified twice by the method of DONALD *et al.* [5]. The pH of the medium was adjusted to 5.5 for *A. tenuis* and to 6.0 for the two species of *Colletotrichum* on the basis of previous experiments.

Four experiments were arranged for each organism. In the first experiment sulphates of zinc, copper and manganese were added to the purified medium at concentrations recommended by STEINBERG [21] for *Aspergillus niger* (*viz.*, zinc, 0.2 µg/ml, copper, 0.03 µg/ml and manganese, 0.02 µg/ml). Solutions containing different concentrations of iron (0.01 µg/ml to 1.0 µg/ml) were prepared with ferric chloride. The response of fungi to iron at these different

concentrations was noted. In the second experiment the concentration of manganese and copper was the same as above. Iron was added at the concentration found to be optimum in the first experiment. In this experiment the response of fungi to different concentrations of zinc was observed. Similarly, the third and fourth experiments aimed at determining the response to different concentrations of copper and manganese, respectively. Separate controls were made for each experiment.

Three replicates were taken and 25 ml of medium was added to 150 ml conical pyrex flasks each and sterilized at 15 lb. pressure for 20 minutes. Each flask was then seeded with 1 ml of standardized spore suspension prepared in purified medium. The inoculated flasks were incubated at 28 °C and fungal mats were harvested at 15 days. The mycelial crop was collected on previously oven-dried and weighed Whatman filter paper No. 40, dried at 60 °C for 3 days, and its dry weight was determined. In general, there was no significant difference between the replicates and hence only average values have been taken into account and used as a quantitative measure for comparing the growth of the fungi under the various treatments. The degree of sporulation was indicated on the basis of the number of spores observed under low power: (1–10) poor; (11–20) fair; (21–30) good; and (above 30) excellent.

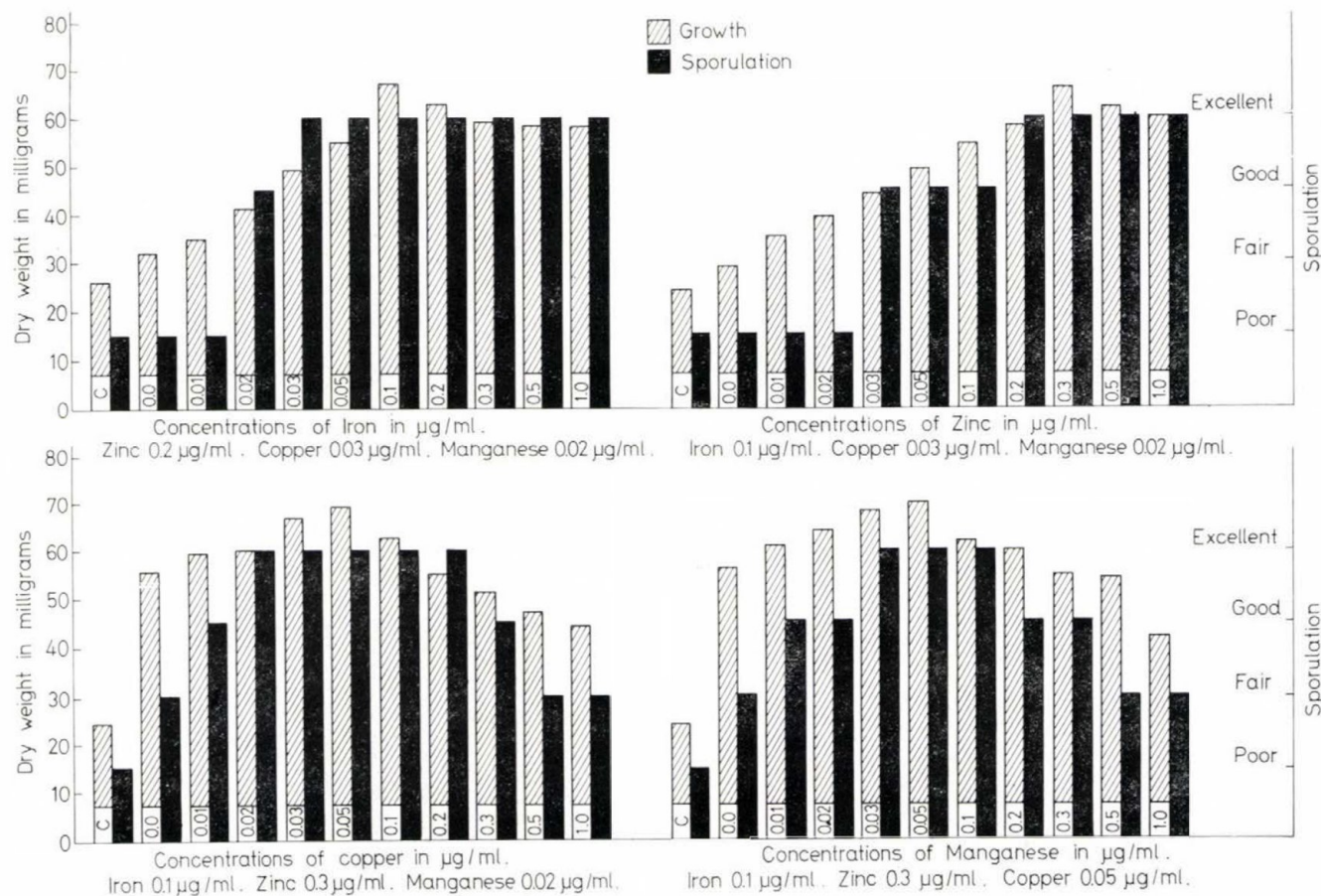
Results

It is clear from the results shown in Figs 1–3 that all the three fungi induced some mycelial growth even in the control medium. There was little difference in growth on a control medium and that obtained on a medium supplemented with all the essential minor elements except iron or zinc. Similarly, the amount of vegetative growth, obtained on media lacking copper or manganese and those supplemented with these trace elements, did not show a marked difference. The dry weight of the organisms increased along with the increasing concentrations of the trace element in question and reached a maximum at a particular concentration after which the dry weight started to decrease. The decrease in mycelial yield was evident at lower concentrations of copper and manganese than of iron and zinc.

The minor elements exerted a marked influence also on the sporulation of the organisms. Sporulation was poor on medium devoid of all the trace elements. In the case of iron and zinc it was also poor on deficient media and at lower concentrations. It changed to fair, good or even excellent at higher concentrations. The sporulation of the two species of *Colletotrichum* was slightly suppressed at higher concentrations of iron and zinc except in *C. gloeosporioides* where the sporulation was found excellent even at 1.0 µg/ml concentration of iron. Sporulation of the pathogens was almost satisfactory on copper or manganese deficient media. On the addition of these elements it increased and reached a maximum at a particular concentration beyond which it became toxic for spore production.

Discussion

The very poor growth and sporulation of the organisms in medium with no copper, iron, manganese and zinc added, probably were due to small amounts of trace elements left in the medium after purification.



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Fig. 1. Growth and sporulation of *Alternaria tenuis* in the presence of different trace elements at various concentrations (C, basal medium)

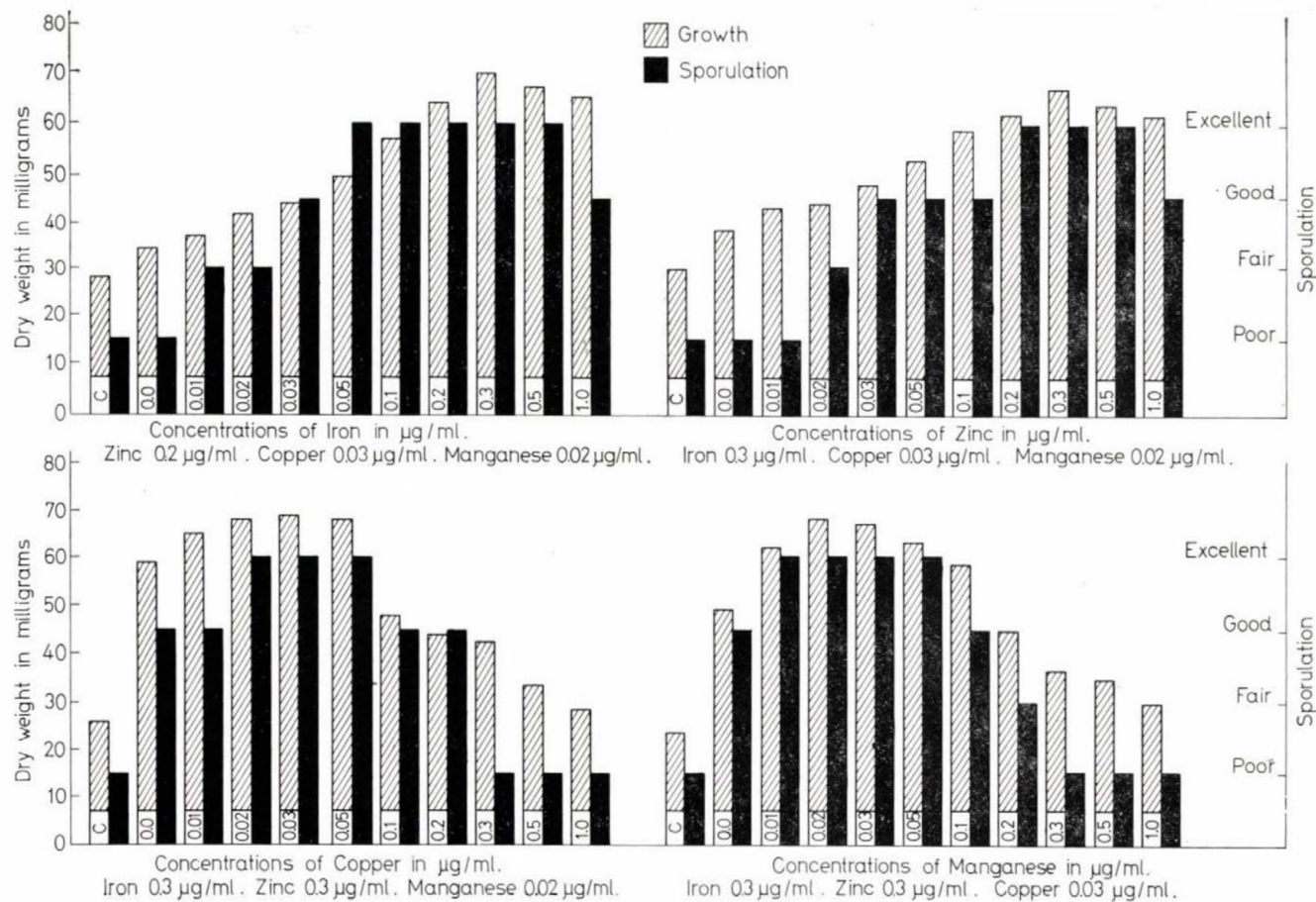


Fig. 2. Growth and sporulation of *Colletotrichum capsici* in the presence of different trace elements at various concentrations (C, basal medium)

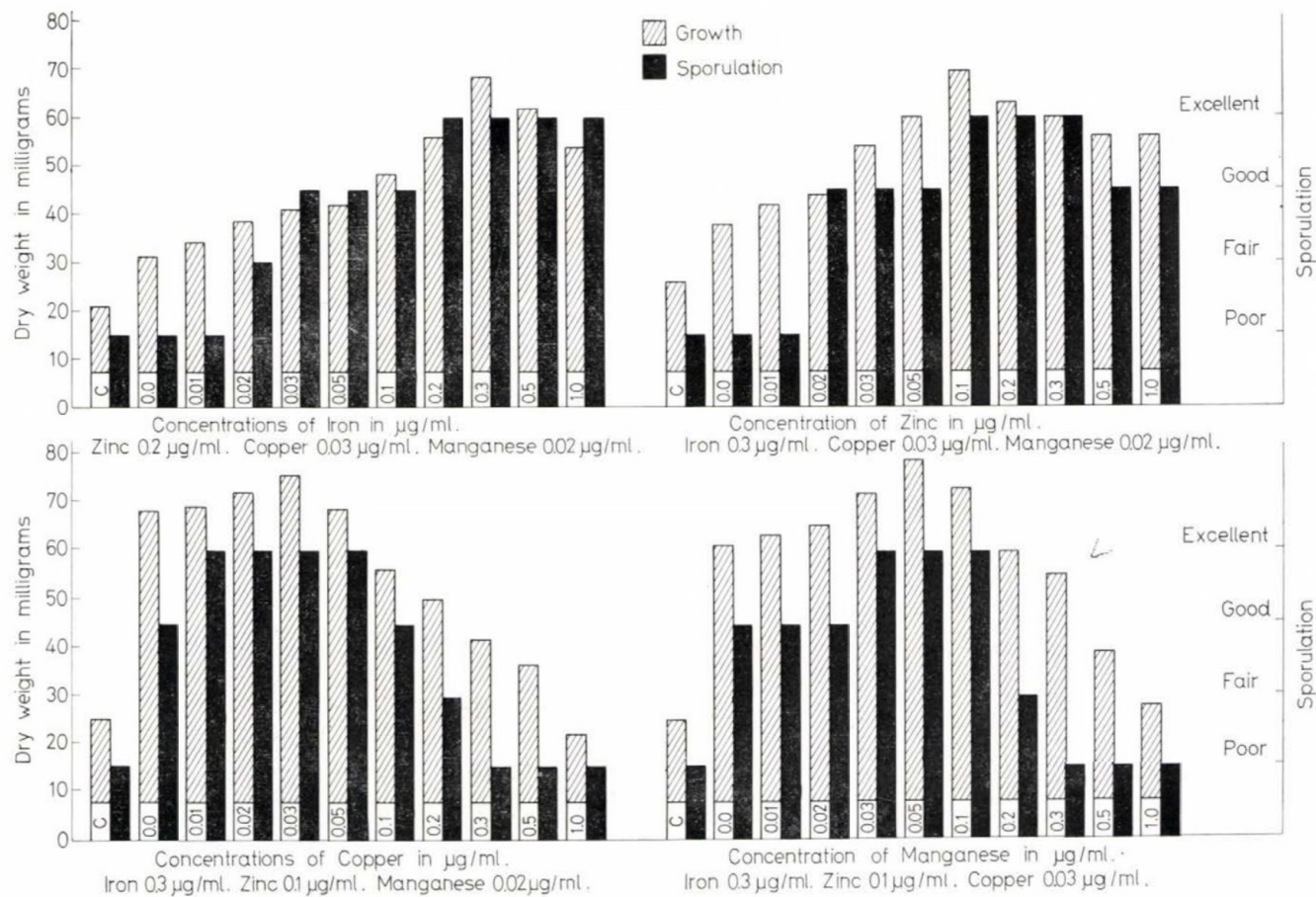


Fig. 3. Growth and sporulation of *Colletotrichum gloeosporioides* in the presence of different trace elements at various concentrations (C, basal medium)

FOSTER [7] stated that in order to test the essentiality of trace elements there should be a significant difference in response between the deficient and supplemented cultures. As in the present investigation there was a significant difference in response of all the three pathogens in iron and zinc-deficient cultures as compared to iron and zinc-supplemented cultures, it may well be surmised that these trace elements are indispensable for the growth of the present pathogens. The present results agree well with those of TANDON [22] and SANKHLA *et al.* [16] who while working with *Alternaria tenuis* and *A. burnsii*, respectively, found reduced growth of the above organisms in the absence of iron and zinc and concluded that these elements were indispensable for normal growth. Similar results have been obtained by several investigators including AGNIHOTRI [1], TANDON and CHANDRA [23], DAFTARI [4] and RAWLA [14]. The essentiality of iron and zinc could easily be explained on the basis that iron is an integral part of some important enzymes like catalase and cytochromes while zinc may be indispensable for the nucleic acid metabolism of fungi. It may also activate various metabolic enzymes like aldolase and dipeptidase. Iron and zinc also had a marked influence on the sporulation. It increased on the addition of these elements and reached a maximum at a certain concentration. The present results are in agreement with those reported by GREWAL [8] for *A. tenuis*, MISRA and MAHMOOD [12] for *Colletotrichum capsici*, BHATNAGAR and PRASAD [3] for *Fusarium solani*.

Surprisingly, in the present investigation the deficiency of copper and manganese in the medium did not seem effectively to reduce the growth of the organisms. HAWKER [9] stated that copper was required only in small amounts and its essentiality was thus difficult to prove. It is quite probable that for normal growth the three fungi in the present investigation needed comparatively smaller traces of copper and manganese as compared to zinc and iron.

SARASWATHI-DEVI [17] working on a number of species of *Fusarium* also reported that copper was needed only in minute quantities. Sporulation of the present organisms on copper or manganese supplemented medium was comparatively better than on copper or manganese deficient medium. These elements at higher concentrations were of course toxic for spore production.

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PERMEABILITY AND CYSTICIDAL ACTION OF EMETINE HYDROCHLORIDE IN THE PRESENCE OF DIFFERENT EXCYSTMENT AGENTS ON THE CYSTS OF SCHIZOPYRENUS RUSSELLI

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Summary. Excystment agents such as *Escherichia coli* extract, glutamic acid, triple glass distilled water, phosphate, carbonate-bicarbonate and Tris buffers failed to cause excystment of *Schizopyrenus russelli* cysts in the presence of emetine hydrochloride. Emetine hydrochloride (1/1000 w/v) in most of the buffers at pH 8.2—9.2 killed 100% of the cysts of *S. russelli*. However, at pH 5.5 emetine in phosphate buffer had hardly any cysticidal effect. L-Glutamic acid (2.0% w/v, pH 6.0) and *E. coli* extract (pH 6.7) failed to cause excystment in the presence of emetine (1/1000 w/v). The treated cysts excysted readily in *E. coli* extract after the removal of emetine. Treatment of emetine in the presence of L-glutamic acid or *E. coli* extract at pH 8—10 killed 100 per cent of the cysts. The aqueous solution of emetine hydrochloride pH 8—10 practically killed all the cysts, but emetine had no adverse effect when the cysts were treated at pH 5.8.

The fragmentary state of our knowledge regarding the physiology of amoebae is clearly reflected in the largely empirical approach in the search for drugs to cure amoebiasis. The frequent relapses in the treated cases of human intestinal amoebiasis suggest the persistence of cysts of *Entamoeba histolytica* which escape the action of drugs *in vivo*. The known amoebicides have little or no effect on the cysts of *E. histolytica*. YORKE and ADAMS [13] found that 5% emetine hydrochloride and Yatren for 30 min *in vitro* had no effect on the cysts of *E. histolytica*. For a rational approach to the chemotherapy of chronic amoebiasis it is important to render the cyst wall permeable to amoebicidal drugs or to prevent the amoebae in forming cysts, or to make them leave the cyst.

Excystment of free living amoebae and *E. histolytica* has been attributed to certain living bacteria (CRUMP [3], DROZANSKI [5], BEERS [1], BUTZEL and HORWITZ [2], DUDZIAK [4], KUNICKI-GOLDFINGER *et al.* [7], SINGH *et al.* [8—11]). SINGH *et al.* [9] have shown that aqueous extracts of *Aerobacter* sp. and *Escherichia coli* cause the excystment of viable sterile cysts of five species of free living amoebae. These findings have been confirmed by DROZANSKI [5]. SINGH *et al.* [12] have shown that both chemically pure D and L amino acids at suitable concentration and pH were responsible for the excystment of cysts of six species of free living amoebae.

This paper reports on the inhibition of excystment of *Schizopyrenus russelli* cysts in the presence of emetine, its cysticidal effect in conjunction with buffers, glutamic acid and *E. coli* extract.

Material and methods

Cysts from a 'pure line' culture of *S. russelli* were used. The amoebae were grown on non-nutrient agar (agar, 2.5%, NaCl, 0.5%, pH 6.8–7.0) plates supplied with a 3–5-day-old culture of *E. coli* as food, grown on nutrient agar slopes. Five to seven-day-old cysts were harvested and viable sterile cysts free from living and dead bacteria were obtained by the method of SINGH *et al.* [12]. The sterility of cysts was tested in nutrient broth and viability was determined by rapid eosin staining, using a 0.125% (w/v) eosin solution in water, and by excystment experiments. Eosin stained the dead cysts within a few seconds while the living ones remained unstained for 5–10 minutes. For excystment experiments, aqueous *E. coli* extract was used. To prepare this extract, the bacteria were killed in chilled acetone, dried, suspended in water, heated at 100 °C for 20 minutes in water bath and centrifuged. The supernatant was about 6.7 pH. The aqueous extract was sterilized at 15 lb/sq. inch pressure for 15 minutes and then dried in a vacuum desiccator; 127 mg of the powder was dissolved in 6.0 ml of distilled water to give a dilution of 1/50. Excystment was studied as described by SINGH *et al.* [9, 11]. A drop of about 0.001 ml of the suspension containing 30–40 cysts was transferred to the centre of a cavity slide and after adding a small drop (0.005–0.01 ml) of test fluid the slide was sealed with a sterile cover glass and put in a moist chamber in a Petri dish. The plates were incubated at 24–25 °C for 24 hours. Percentage excystment was calculated from the count of the amoebae and the unexcysted cysts. A cyst was considered excysted only when an amoeba had escaped from it and was moving freely in the surrounding medium.

Cysts were treated either with buffers, glutamic acid, *E. coli* extract and distilled water +1/1000 (w/v) aqueous emetine HCl at different pH for 48 hours, or with excystment agents followed by emetine hydrochloride treatment. In both cases, after 48 hours, the emetine was washed off completely and the treated cysts were excysted with *E. coli* extract to check the viability of the cysts. To ascertain whether sterile conditions had prevailed during the experiment, the fluid from the cavity slides was drawn out at the end of the experiment and inoculated into nutrient broth or on nutrient agar.

The K_2HPO_4 , KH_2PO_4 , K_2CO_3 , $KHCO_3$, Na_2CO_3 , $NaHCO_3$, Na_2HPO_4 , NaH_2PO_4 and tris (hydroxymethyl)-amino-methane used were B. D. H. products; HCl and trypsin were made by Merck and the glutamic acid used was obtained from Aldrich Chemical Co. Inc., Milwaukee, U.S.A., and the emetine hydrochloride from Burroughs Wellcome and Co., London.

Results

About 40–60% of cysts of *S. russelli* excysted in M/10 phosphate, carbonate, bicarbonate, and tris buffer at pH 8.2–9.2 [12]. Cysts of *S. russelli* when treated with emetine (1/1000 w/v) in buffers at pH 8.2–9.2, either completely failed to excyst in *E. coli* extract or there was hardly any excystment after the emetine and buffers had been washed off completely (Table I). This suggests that at alkaline pH, the buffers render the cyst wall permeable to emetine and the latter is killing the cysts. Emetine in buffer had hardly any effect on *S. russelli* cysts at pH 5.5. Similarly, cysts treated with emetine in distilled water at pH 5.8 (control) readily excysted in *E. coli* extract after the removal of emetine. This clearly shows that emetine is unable to enter the cyst at an acid pH. It has been shown earlier by SINGH *et al.* [9, 11, 12] that an aqueous extract of *Aerobacter* sp., *E. coli* and certain amino acids failed to excyst the cysts of *S. russelli* at pH 2.5–5. In the present investigation it has been found that emetine (1/1000 w/v) together with *E. coli* extract (1/400) and L-glutamic acid (2% w/v) had a lethal effect on the cysts of *S. russelli* at pH 7.0–10.5. These cysts when treated with emetine (1/1000 w/v) at pH

Table I

Permeability and cysticidal action of emetine hydrochloride on cysts of S. russelli in conjunction with buffers at different pH*

Treatment	pH	No. of cysts	No. of cysts excysted	Per cent excystment
1. $\text{NaH}_2\text{PO}_4 + \text{Na}_2\text{HPO}_4$ (M/10) + emetine (1/1000)	5.5	147	109	74
	7.2	867	385	44
	8.2	730	—	—
2. $\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$ (M/10) + emetine (1/1000)	5.5	177	147	83
	7.2	448	191	43
	8.2	514	—	—
3. Tris (M/10) + emetine (1/1000)	7.2	434	43	10
	8.2	497	—	—
4. $\text{Na}_2\text{CO}_3 + \text{NaHCO}_3$ (M/10) + emetine (1/1000)	9.2	213	8	4
5. $\text{K}_2\text{CO}_3 + \text{KHCO}_3$ (M/10) + emetine (1/1000)	9.2	164	1	2
6. Control; emetine (1/1000) in distilled water	5.8	530	469	88

* Cysts were treated with buffers and emetine together for 48 hours and excystment was determined in *E. coli* extract after washing the cysts with distilled water to remove the emetine

Table II

Cysticidal effect of emetine hydrochloride on cysts of S. russelli at alkaline pH in the presence of L-glutamic acid*

Treatment	pH	No. of cysts	No. of cysts excysted	Per cent excystment
L-Glutamic acid (2%) + emetine (1/1000)	6.4	218	205	94
	7.0	88	13	14
	7.5	137	13	10
	8.0	91	—	—
	8.5	137	1	1
	9.0	130	—	—
	9.5	130	—	—
	10.0	181	—	—
Control; emetine (1/1000) in distilled water	5.8	290	271	93

* Cysts were treated for 48 hours and excystment was determined in *E. coli* extract after washing the cysts with distilled water to remove glutamic acid and emetine

Table III

Permeability and cysticidal action of emetine hydrochloride on cysts of S. russelli in the presence of E. coli extract at different pH*

Treatment	pH	No. of cysts	No. of cysts excysted	Per cent excystment
<i>E. coli</i> extract (1/400) + emetine (1/1000)	4.5	23	22	96
	5.0	22	21	95
	5.5	47	44	94
	6.0	79	74	94
	6.5	189	167	88
	7.0	207	42	20
	7.5	166	2	1
	8.0	177	—	—
	8.5	18	—	—
	9.0	51	—	—
	9.5	72	—	—
	10.0	88	—	—
	10.5	63	—	—
Control: emetine (1/1000) in distilled water	5.8	230	217	94

* Cysts were treated with *E. coli* extract and emetine for 48 hours and excystment was determined in *E. coli* extract after thorough washing of the cysts with distilled water to remove emetine + *E. coli* extract

Table IV

Permeability and cysticidal action of emetine hydrochloride on cysts of S. russelli in triple glass distilled water at different pH*

Treatment	pH	Excystment by <i>E. coli</i> extract			Viability by eosin staining		
		No. of cysts	No. of cysts excysted	Per cent excystment	No. of cysts	No. of unstained cysts	Per cent viability
Emetine (1/1000) in distilled water	4.0	273	231	85	73	65	89
	4.5	404	335	83	91	85	93
	5.0	237	196	83	99	93	95
	5.5	248	214	86	94	89	95
	6.0	102	84	82	108	88	81
	6.5	124	95	77	122	96	79
	7.0	292	220	75	115	88	77
	7.5	211	127	60	114	79	69
	8.0	207	—	—	94	10	11
	8.5	197	—	—	50	8	6
	9.0	328	—	—	68	—	—
	9.5	321	—	—	72	—	—
	10.0	128	—	—	98	—	—

* Cysts were treated for 48 hours and excystment was determined in *E. coli* extract and their viability by eosin staining after thorough washing of the cysts with distilled water to remove emetine hydrochloride

4.5–6.5 in the presence of L-glutamic acid and *E. coli* extract were hardly affected by emetine and about 94% of the cysts excysted when the emetine had been washed off completely (Tables II and III). Emetine (1/1000 w/v) in triple glass distilled water at pH 8.0 to 10.0 also killed 100% of the cysts of *S. russelli* (Table IV).

The cysts of amoebae killed at alkaline pH by emetine in buffers, L-glutamic acid, *E. coli* extract and distilled water showed a contracted protoplasm and stained readily with eosin.

Discussion

It has been shown in the present investigation that excystment-inducing agents like buffers, L-glutamic acid and *E. coli* extract failed to cause excystment of *S. russelli* cysts in the presence of emetine hydrochloride and caused 100% killing of the cysts at pH 7.5–10.5. IMAM *et al.*, [6] have reported that excystment agents like L-glutamic acid (2.0% w/v; pH 6.4) and *E. coli* extract (pH 6.5) failed to cause excystment of cysts of *S. russelli* in the presence of emetine (1/1000 w/v). This lethal effect of emetine is probably due to the loose binding between emetine and the excystment agents. In the present study it was observed that emetine at an acid pH was unable to kill the cysts, although it prevented the excystment caused by *E. coli* extract or L-glutamic acid, as reported earlier by IMAM *et al.* [6]. This loose binding between emetine and excystment agents becomes ineffective at alkaline pH, as is shown by the data in Tables I, II, III and IV. This clearly shows that the cyst wall becomes permeable to emetine hydrochloride at an alkaline pH in the case of all the excystment agents. These findings further reveal that the pH plays an important role in the excystment of cysts of amoebae and also in rendering the cyst wall permeable to amoebicidal agents. These findings may find an application in the eradication of the cysts of *E. histolytica* in human carriers.

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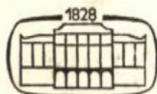
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A COMPARATIVE STUDY ON SHIGELLA FLEXNERI CONVERTING PHAGES

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Summary. (i) The studied antigen converting phages of *Shigella flexneri* showed the same electron microscopic morphology, belonged to the group A of BRADLEY, having an octahedral head and a long contractile tail. Their plaque morphology as well as several of their biochemical and biological properties were also identical.

(ii) A special group of the strains lysogenic with these phages was formed by lysogenic convertants. Dilyso-genic convertants could be produced with any of the phages. Converted antigens IV₁, V, and 7,8 may be expressed synchronously while antigen I only with antigen 7,8.

(iii) The converting phages exhibited similar host ranges and they proved to be closely related also in cross-neutralization tests. As to immunity, they formed a homoimmune system, but a reduced repressor production depending on the phage and host-cell was observed in some instances in the converting group of the lysogenic prophages.

There are several bacteriophages known to induce lysogenic conversion expressed in the so-called type specific antigens of *Shigella flexneri*. The first of these phages converting into antigen specificity IV₁ was described by MATSUI [1]. Later, two phages with identical converting properties were isolated by ISEKI and HAMANO [2]. The conversion presented in our previous paper [3] was only partially identical with that of antigen specificity IV₁. OKADA *et al.* [4] described a phage designated φ_1 which proved the lysogenic origin of antigen I. GIAMMANCO [5] and GIAMMANCO and NATOLI [6] reported on the isolation of phages converting antigen II, antigen 7,8 group-specific in the diagnostic sense, and antigen V. In the present paper, observations on a phage isolated by us and also converting type V-specific antigen are presented.

The common characteristics of these phages are that all of them are temperate non UV-inducible phages isolated from lysogenic strains of *Sh. flexneri*, that the genetic information for the antigen converted by them may be localized in the *pro-lac* region [7, 8], and that specific UDP-glucose-transferase enzymes are the mediators of the conversion as shown by SIMMONS [9, 10]. This incompleteness of the data available has prompted us to perform a comparative study of these phages.

Materials and methods

Bacterial and phage strains. The strains of *Sh. flexneri* used are shown in Table I. The numerous lysogenic and dilyso-genic convertants derived from strains UP4204, UP511, UP111, and NTCC 4839 are not included in the Table, neither are included the few derivatives treated with anti-phage sera. The standard type strains of *Sh. flexneri* used have been described previously [3]. The more important data of the phages studied are shown in Table II.

Media. For cultivation of the strains and convertants, a meat broth containing 0.5% NaCl and 1% peptone and an agar medium prepared by adding 1.8% agar-agar to the meat broth, were used.

The overlayer used for phage titration contained 0.6% agar-agar.

Table I
Lysogenic and relevator strains

Strain		Lysogenic	Relevator
designation	serotype		for the given phage
UP90	4b	P90	—
4c var.	4b	φ_{IV_1}	—
1a var.	1a	φ_1	—
EW 595/52	5	fV	—
UPE	2a	PE5	—
NTCC 4	2b	f7,8	—
UP511	1a	—	P90, φ_{IV_1} , φ_1 , f7,8, fV, PE5
UP111	2a	—	φ_1
NTCC 4839	var. Y	—	φ_{IV_1} , φ_1 , f7,8, fV, PE5
UP4204	4b (IV ₁ ⁻)	—	Hfr, Nia ⁻

Table II
Sh. flexneri converting phages

Phage	Strain lysogenic with the phage		Relevator strain	Antigen converted	Origin of the phage
	Designation	Serotype			
P90	UP90	4b	UP511	(IV ₁)*	Own isolation
φ_{IV_1}	4c var.	4b	UP511	IV ₁	Dr. S. ISEKI
φ_1	1a var.	1a	UP111	I	Dr. S. ISEKI
f7,8	NTCC 4	2b	NTCC 4839	7,8	Dr. G. GIAMMANCO
fV	EW 595/52	5	NTCC 4839	V	Dr. G. GIAMMANCO
PE5	UPE	2a	NTCC 4839	V	Own isolation

* = in an a, b—a, c antigenic relation with antigen IV₁

Isolation of phages from lysogenic strains. The lysogenic strains were plated on agar media and after an incubation at 37°C for 18–24 hours the cultures were killed in chloroform vapour (15-minute treatment followed by evaporation for one hour). An 18-hour bouillon culture of the relevator strain (0.3 ml) was then added to 3 ml soft agar (0.6%) and overlaid by the method of ADAMS [11]. Next day, a single plaque was transferred into a few ml of broth. The bacteria were killed with chloroform, centrifuged, and then plates were made from dilutions of the supernatant. Subsequent isolation in triplicate of a single plaque guaranteed a pure phage strain for further experiments. None of the phages was found UV-inducible.

Preparation of phage lysates. Each of the agar cultures showing confluent or near confluent lysis was washed off with 3 ml bouillon using glass rods to expose the overlayers. The bacteria killed by chloroform as well as the detritus were removed from the lysates by centrifugation at 7–8000 g.

Titration of phages. The titres were determined by the overlayer method. Results were expressed in terms of e.o.p. (efficiency or efficiencies of plating) or of relative e.o.p. values. For relevator strains, these were equal to the PFU/ml (plaque forming unit) values.

Lysogenization and lysogenic conversion. For lysogenization, phage lysates of known titres and host-cells of known germ count were inoculated into 5 ml aliquots of bouillon. After 24 hours incubation at 37°C, samples were plated and the phage resistance of 100 colonies was scored.

The strain designed for lysogenic conversion was subcultured serially at least 5 times in bouillon containing 10^8 – 10^{10} PFU/ml phages. After the last passage, the samples were plated on agar. At least 50 colonies were isolated. Convertants as well as the rate of conversion were detected by slide-agglutination using adequate factor sera. Lysogenic conversion was considered lacking only if 10 subcultures and the scoring of a hundred colonies gave negative results.

Production of antiphage sera. Rabbits were injected with increasing doses (0.2, 0.5, 1.0, 1.0, 2.0, 2.0, and 5.0 ml) of high titre phage lysates twice weekly for 3–4 weeks. After the last dose, the animals were bled through the carotid. The sera were separated by centrifugation, sterilized by filtering through Seitz filter and stored in small frozen aliquots.

The lysates were prepared from strain NTCC 4839 (var. Y). In order to avoid interfering anti-bacterial antibody effects, strain UP511 was used as indicator in neutralization tests.

Neutralization tests. Aliquots of 0.1 ml phage lysates of titres 10^7 PFU/ml were added to 0.9 ml of each of the 10, 100 and 1000-fold dilutions of the sera. The mixture of the pre-heated components was incubated at 37°C for 5 and 15 minutes, as suggested by preliminary experiments. The samples were diluted immediately 1 : 100 and the e.o.p. was determined. In order to facilitate comparison, the results were given in terms of relative "K" values taking into account the titre of the non-neutralized phage titrated in parallel.

Antiphage serum treatment. The lysogenic convertant was subcultured 3–5 times in bouillon containing antiphage serum in a dilution of 1 : 10. Evaluation of the effect of antiphage sera was based on the antigenic structures studied by slide-agglutination of 20–50 colonies isolated after plating [28].

Phage adsorption. During these informatory experiments, a log phase bacterial culture containing 10^7 germs and a phage suspension of 10^9 – 10^{10} PFU/ml were inoculated into bouillon preheated to 37°C. The samples taken at 5 minute intervals were diluted with ice-cold bouillon containing chloroform (10 : 1). After centrifugation, the supernatant was used for determination of the e.o.p. As in the neutralization tests, "K" values were calculated only at the levels of 90 and 99%.

Serological studies. Factor sera in 1 : 10 dilution and slide-agglutination were used. The results thus obtained were confirmed by antigen absorption studies.

Electron microscopic studies. Lysates of high titre purified by repeated centrifugation were dropped on grids covered by carbon film. The excess lysate was removed with filter paper. A 2% neutral solution of phosphorous tungstic acid (PTA) was then added and its excess was removed with filter paper. The wet preparations were studied by a TESLA electron microscope, type BS 613, at an original magnification of 40 000–60 000.

Estimation of nucleic acids. Nucleic acids were estimated on the basis of their acridine orange fluorescence by the method of BRADLEY [12]. One drop of the high titre lysate in borate buffer (pH 9.0) was dried on a coverslip. The preparation was fixed in Carnoy's fluid for 5 minutes, dehydrated with absolute ethanol, and stained with 0.01% acridine orange. After two washings in McIlvaine buffer (pH 3.8), the coverslips were placed into 0.15 M Na_2HPO_4 for 15 minutes. Finally, the fluorescence of the preparation was studied under a low-pressure mercury-vapour lamp. For further differentiation, treatments, with molybdenic acid, tartaric acid, DNase (0.02%), and RNase (0.1%) were applied.

Results

1. Converting phages and their isolation

Phage P90. Isolation of this phage and a comparative study of this and of phage φ_{IV_1} were described previously [3]. The phage was carried by strain "90" of the serotype 4b of *Sh. flexneri*. In the present paper some further data on this phage are given.

Phage φ_{IV_1} . A lysogenic strain (var. 4c) of this phage was kindly provided by Dr. S. ISEKI. Strains UP511, NTCC 4839, and others, also proved to be competent as relevelators.

Phage φ_1 . This strain as the lysogenic strain "1a var." was also kindly provided by Dr. S. ISEKI. For its propagation and as indicator mainly strain UP111 (2a) served, but strain NTCC 4839 was also competent.

Phage f7,8. Upon our request, the lysogenic strain NTCC 4 of the serotype 2b and the relevelator strain NTCC 4839 (var. Y) were kindly sent to our laboratory by Dr. G. GIAMMANCO. Phage f7,8 was successfully isolated and propagated, but attempts at isolating phage fII have failed. Neither could be phages of the same characteristics isolated from other strains of the serotypes 2a and 2b. Consequently, phage fII as another converting phage [5] could not be studied in these comparative investigations.

Phage fV. This phage was isolated and propagated on lysogenic strain EW 595/52 of *Sh. flexneri* serotype 5 kindly provided by Dr. G. GIAMMANCO. The strain of NTCC 4839 served also as relevelator culture.

Phage PE5. Our attempts at isolating a phage with fII characteristics resulted in the isolation of a temperate phage from the strain E of *Sh. flexneri* serotype 2a. This phage induced V antigenic conversion in strains UP511 (1a), NTCC 4839 (var. Y), and UP 4204 (4b) with an unexpectedly high frequency. This finding was supported by the following: isolation of a phage of similar characteristics from the same UPE strains was repeatedly successful, strain UPE showed an immunity against the phage denoted PE5, phage PE5 (as well as phage fV) failed to induce lysogenic conversion in strain UPE (and in strain UP111 2a) and, finally, derepressed clones against phage PE5 were obtained after treatment of strain UPE with phage PE5 anti-serum.

Each of the above phages was characterized by a resistance against chloroform, a low stability in physiological salt solution, a moderate stability in borate buffer (pH 9.0) and by a lack of UV inducibility.

2. Morphological and biochemical studies

(a) *Electron-microscopy.* These informatory studies showed that all the 6 phages studied (P90, φ_{IV_1} , φ_1 , f7,8, fV and PE5) had the same morphological characteristics and were of the same size. As measured by random rotation, the octahedral head was about $60-70 \times 60-70$ nm in size (Figs 1a





Fig. 1. Electron microscopic morphology of *Sh. flexneri* converting phages. Electron micrographs taken by a TESLA BS 613 electron-microscope (PTA): a: phage PE5 ($\times 330\,000$); b: phage φ_{IV_1} ($\times 240\,000$); c: phage f7,8 showing contracted sheath ($\times 240\,000$); d: phage f7,8 showing contracted sheath and empty head ($\times 240\,000$)

and b), the contractile straight tail attached to the head with a neck, but without any discernible collar (Figs 1a and d). The long tail, measuring 90–100 nm in length, showed a helical structure and appeared in some of the pictures to end in a basal plate-like structure. It could not, however, be decided whether there were indeed organelles attached to the basal plate and if so whether these corresponded to basal filaments (Figs 1b and c) or spikes (Fig. 1a). At any rate, besides their identity in size and essential morphology, our phages belonged to BRADLEY's group A [13].

(b) *Plaque morphology.* As to plaque morphology, no differences could be demonstrated between the phages studied. The plaques formed showed the characteristics of minute and clear plaques. In some of them, mainly in the larger ones, halo formation was observed after prolonged incubation. Conspicuous were the differences in size, known to result mainly from a slow absorption of the phage with the technique of immediate plate pouring (Fig. 2). This assumption was supported by the results of our experiments on the rate of absorption of phage PE5 to NTCC 4839 host-cells when a "K" value of 2.8×10^{-10} was found.

(c) *Phage nucleic acids.* The phages described thus far to belong to BRADLEY's A, B and C groups are known to consist of double-stranded DNA. Of our phages known to belong to BRADLEY's group A, only phages PE5 and φ_{IV_1} were studied for nucleic acids. Using the method of BRADLEY [12], preparations stained with acridine orange showed a bright green fluorescence which persisted after treatment with molybdenic acid, tartaric acid, and RNase, but disappeared after treatment with DNase. Thus, as expected, these phages contained double-stranded DNA.

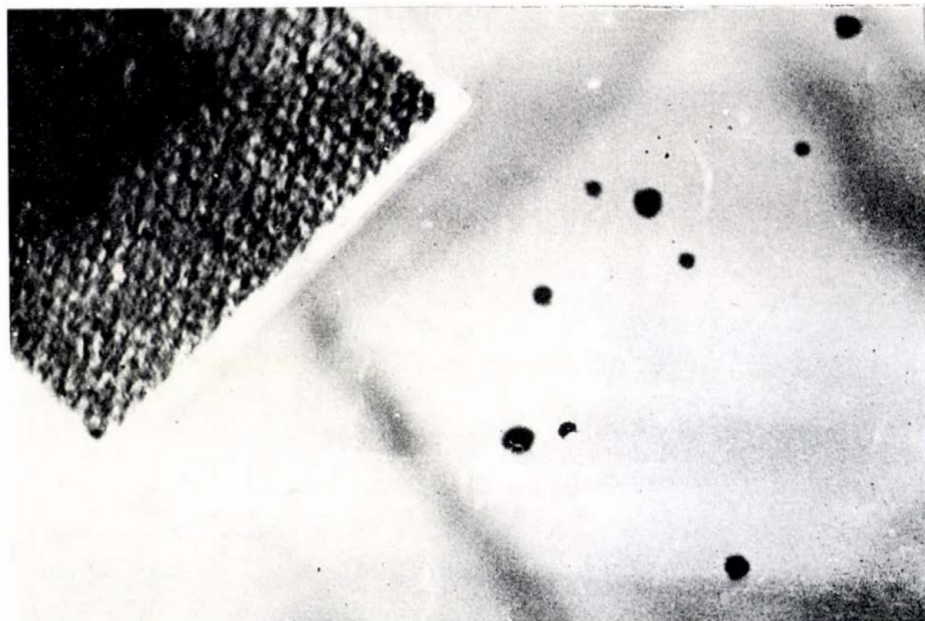


Fig. 2. Plaque morphology of *Sh. flexneri* converting phages. Plaques induced by phage PE5 on relevarator strain UP511 ($\times 5$); overlayer method, using 0.6% agar-agar; plaque diameter, 0.3–0.8 mm

3. Lysogenic conversion

In the experiments on lysogenic conversion, our Hfr strain UP 4204 of *Sh. flexneri* 4b [14] was chosen as host-cell with a purpose of using it as donor in localization studies. The strain was successfully converted by all our phages except phage f7,8 after 5 subcultures in bouillon containing the phages as detected by slide-agglutination on the basis of the appearance of IV₁ (φ_{IV_1} and P90), I (φ_1), and V (fV and PE5) antigen specificities (Table III). Strain UP511 (1a) used in earlier studies [3] was converted by all phages including phage f7,8, except phage φ_1 . Strain NTCC 4839 (var. Y) was converted by each of our phages while the conversion of strain UP111 (2a) used as a provisory relevarator strain for phage φ_1 failed with phages fV and PE5.

As the next step, preparation of dilysogenic derivatives was attempted in order to study a possible occurrence of interference between our phages. The results of these experiments are summarized in Table IV. The data showed that either pair of the phages may produce dilysogenic derivatives, irrespective of which of the phages is playing the role of primary convertant. In one instance (not included in Table IV) a triple lysogenic convertant of phages P90, φ_{IV_1} , and fV was produced. The "secondary" conversion of the convertants occurred with a frequency not lower than that of either of the "primary"

Table III

Lysogenic conversion with Sh. flexneri converting phages

Phage	Antigen—conversion ratio in indicator strain			
	UP4204	UP511	UP111	NTCC 4839
P90	11/50*	3/50	.**	2/50
φ_{IV_1}	23/50	46/50	.	7/50
φ_I	3/50	.	11/50	2/50
f7,8	—***	10/50	.	9/50
fV	8/50	40/50	—	4/50
PE5	42/50	37/50	—	19/50

* Number of colonies converted/studied

** Not studied

*** Conversion experiment unsuccessful

Table IV

Production of dilysoogenic convertants

Phage	"Primary" lysogenic convertants					
	P90	φ_{IV_1}	φ_I	f7,8	fV	PE5
P90	.	+	.	.	+	.
φ_{IV_1}	+	.	+	+	+	+
φ_I	.	+	.	+	+	+
f7,8	.	+	+	.	+	+
fV	+	+	+	+	.	.
PE5	.	+	+	+	.	.

. = Not studied

+ = "Secondary" lysogenic conversion successful

Note. As host-cells, the following strains were used: NTCC 4839 (var. Y), UP 511 (1a) except for phage φ_I , and UP4204 except for phage f7,8

conversions. Due to the expression of the same antigenic specificity, the interference between phages fV and PE5 could not be studied.

When studied by slide-agglutination, the simultaneous occurrence of some of the antigen specificities as well as the disappearance of some of the "primarily" converted antigen specificities could be observed in the double convertants. Relevant data presented in Table V show that antigen specificity 7,8 may occur parallel to antigens I, IV_1 or V also originating from lysogenic conversion. Antigens IV_1 and V may likewise be expressed simultaneously. On the contrary, the effects of phages converting antigens IV_1 or V result

Table V

Serological studies on mono and dilysogenic convertants

Phage converting secondarily	Antigenic structure of lysogenic convertants*				
	φ_{IV_1}	φ_I	7,8	fV	PE5
None	IV ₁	I	7,8	V	V
φ_{IV_1}	.	IV ₁	7,8 : IV ₁	V : IV ₁	V : IV ₁
φ_I	I	.	7,8 : I	I	I
f7,8	IV : 7,8	I : 7,8	.	V : 7,8	V : 7,8
fV	IV ₁ : V	V	7,8 : V	.	.
PE5	IV ₁ : V	V	7,8 : V	.	.

* = Showing only antigens converted on strains NTCC 4839, UP511 except for phage φ_I , and UP4204 except for phage f7,8. Slide-agglutination tests

. = Cannot be studied

Table VI

Antigenic absorption of antigens originating from conversion

Convertants used for absorption	Antibody factors			
	IV ₁	I	7,8	V
φ_{IV_1} (IV ₁)*	+	.	.	.
$\varphi_I + \varphi_{IV_1}$ (IV ₁)	+	—	.	.
$\varphi_{IV_1} + \varphi_I$ (I)	—	+	.	.
$\varphi_{IV_1} + fV$ (IV ₁ + V)	+	.	.	+
$\varphi_{IV_1} + f7,8$ (IV ₁ + 7,8)	+	.	+	.
φ_I (I)	.	+	.	.
f7,8 + φ_I (7,8 + I)	.	+	+	.
fV (V)	.	.	.	+
PE5 (V)	.	.	.	+
fV + φ_{IV_1} (V + IV ₁)	+	.	.	+
PE5 + f7,8 (V + 7,8)	.	.	+	+
f7,8 (7,8)	.	.	+	.
f7,8 + φ_{IV_1} (7,8 + IV ₁)	+	.	+	.
f7,8 + φ_I (7,8 + I)	.	+	+	.
f7,8 + fV (7,8 + V)	.	.	+	+

* The convertant is symbolized by the phage and, in brackets, by the antigen expressed

+ = Absorption of the studied factor

— = Lack of absorption

. = Not studied

in the disappearance of antigen I originating from conversion. The same occurs in reciprocal experiments.

The antigen absorption data summarized in Table VI not only supported the results of slide-agglutination experiments, but also threw light on the fate of the antigens which "disappeared" during "secondary" conversion. Data in the second and third rows of Table VI show that the not expressed antigens I and IV₁ exhibit no capacity of absorption, viz., no masking effect due to antigens I and IV₁.

It is of course possible that the secondary converting phage promotes or induces the exclusion of the primary converting phage. In other words,

Table VII

*Effects of antiphage-sera on antigens not expressed
in dilysonic convertants*

Phages carried by the convertants listed in the order of lysogenization	Antigen expressed	Antiphage- serum treatment	Agglutination in factor-sera		
			IV ₁	I	V
$\varphi_{IV_1} + \varphi_I$	I	0	—	+++	.
		φ_I	++	—	.
$\varphi_I + \varphi_{IV_1}$	IV ₁	0	+++	—	.
		φ_{IV_1}	++	++	.
fV + φ_I	I	0	.	+++	—
		φ_I	.	++	++
PE5 + φ_I	I	0	.	+++	—
		φ_I	.	++	++
φ_I + fV	V	0	.	—	+++
		fV	.	++	++
φ_I + PE5	V	0	.	—	+++
		PE5	.	++	++

0 = Without treatment with phage anti-serum

++, +++ = Positive slide-agglutination

— = Negative reaction

. = Not studied

these convertants would be monolysogenic. In order to test this hypothesis, the strains were treated with antiphage sera of expressed phage. As shown in Table VII, antigen specificity converted primarily has appeared in every strain proving the presence of these prophages and the intactness of their converting function. In most of these cases, however, the simultaneous occurrence of the two antigen specificities — thus far excluding each other — could be observed (Table VII). For the time being, no explanation of this finding can be offered. Our attempts to demonstrate a role of bifunctional recombinant phages in dilysogenic conversion have failed.

Table VIII

Host range of Sh. flexneri converting phages

<i>Sh. flexneri</i>		Phage sensitivity					
types	strains	P90	φ IV ₁	φ I	f7,8	fV	PE5
1a	UP511	+	+	+	+	+	+
1b	UP1000	+	+	+	—	—	+
2a	UP512	—	+	+	+	+	+
2b	UP526	—	+	+	+	+	+
3a	UP513	+	+	+	+	+	+
3b	UP9124	—	—	+	+	+	+
4aA	UP536	—	—	—	—	—	—
4aB	UP537	—	—	—	—	—	—
4b	UP90	—	+	+	—	—	—
5	UP515	—	—	—	—	—	—
6	UP516	—	—	—	—	—	—
var. X	UP517	—	—	—	+	+	+
var. Y	UP518	+	+	+	+	+	+

+ = Lysis
 -- = No lysis

4. Biological homogeneity of the *Sh. flexneri* converting phages

(a) *Host range.* For the determination of the host range of the phages on the basis of lytic capacity, only the type strains of *Sh. flexneri* were used. In informatory studies *E. coli* K-12, B and C and *S. typhi-murium* LT2 proved resistant against the phages studied. The susceptibility of our *Sh. flexneri* type strains is shown in Table VIII. The phages studied differed slightly in their host range so that phages f7,8 and fV, for instance, could not be distinguished by the strains used.

(b) *Phage neutralization.* For neutralization tests, phage lysates of high titre and antiphage sera prepared with the lysates were used. The absolute

values for "K" varied between 15.2 and 96. To allow a comparison, Table IX presents relative "K" values, taking the "K" values obtained in homologous neutralization tests as "100". As shown in Table IX, antiphage sera exhibited high cross-neutralization values indicating a serological involvement of only a limited part of the protein envelope of the phage. Phage P90 which in this respect was found identical to phage φ_{IV_1} [3] has not been included in these studies.

Table IX
Phage neutralization tests

Phages	Relative "K" values of antiphage-sera				
	φ_{IV_1}	φ_I	f7,8	fV	PE5
φ_{IV_1}	100	100	50	100	10
φ_I	100	100	100	100	10
f7,8	50	100	100	50	10
fV	50	100	100	100	100
PE5	30	100	50	100	100

Note. The absolute value for "K" varied between 15.2 and 92; relative value of 100 = relative K-value of the homologous phage; the relative figures of 100, 50, 30, and 10 for the heterologous phages represent relative neutralization values within the limits of error.

(c) *Phage immunity and repressor sensitivity.* Strain NTCC 4839 (var. Y) and its lysogenic convertants were first studied since they were susceptible to, and convertible with, all of the phages used. However, as the estimation of the e.o.p. for phage P90 revealed a reduction of 10^{-7} , the experiments were repeated with strain UP511 (1a) with the omission of phage φ_I . The results are shown in Table X.

It has been concluded that each of the prophages present in the lysogenic convertants represses heterologous phages, *viz.*, the phages studied belonged to a homoimmune system. In contrast, changes were observed in the repressor production of certain phages depending on the host-cells. In NTCC 4839 (var. Y), prophage f7,8 produced a reduced amount of repressor (0.01–0.1). The φ_I -repressor sensitivity of prophage fV was decreased in the same host-cell. Using strain UP511 (1a) as host-cells in the same experiments, only prophages P90 and φ_{IV_1} showed a reduced repressor production (0.1–0.3 and 0.005–0.1, respectively) while prophage f7,8 failed to exhibit this characteristic phenomenon.

A satisfactory explanation of this finding presupposes the knowledge of phage and bacterial genomes. Nevertheless, the following working hypothesis has been made. During lysogenic conversion, an insertional-recombina-

Table X
Phage immunity

Phage	Lysogenic convertants of strain NTCC 4839 (relative e.o.p.)						
	Control	(P90)	(φ_{IV_1})	(φ_I)	(f7,8)	(fV)	(PE5)
P90	1*	—	—	—	—	—	—
φ_{IV_1}	1	—	—	—	0.1	—	—
φ_I	1	—	—	—	1	—	—
f7,8	1	—	—	—	0.1	—	—
fV	1	—	—	0.1	0.01	—	—
PE5	1	—	—	—	0.05	—	—
Phage	Lysogenic convertants of strain UP511 (1a)						
	Control	(P90)	(φ_{IV_1})	(φ_I)	(f7,8)	(fV)	(PE5)
P90	1	0.3	—	.	—	—	—
φ_{IV_1}	1	1	0.04	.	—	—	—
φ_I	1	0.4	0.1	.	—	—	—
f7,8	1	1	0.1	.	—	—	—
fV	1	1	0.02	.	—	—	—
PE5	1	1	0.005	.	—	—	—

1* = On this strain, phage P90 results in reduced e.o.p. (10^{-7})

1 = 10^9 – 10^{11} /ml e.o.p.; 0.1–0.005 = relative reduction of e.o.p.

— = No plaques detectable even with 1 : 10 dilution of lysate

. = Cannot be studied

tional event occurs between the *att* regions which may influence the control of repressor production (a reduced repressor production indicates a positive control). A possibility to separate lysogeny from the lysogenic condition of conversion function would support our working hypothesis and at the same time indicate immunological differences.

In these preliminary experiments, phage PE5 was studied first. After 24 hours incubation of the phage lysate (titre: 10^{10} PFU/ml) with strain NTCC 4839 (10^7 /ml germs in 5 ml bouillon at 37°C) the conversion and PE5 phage resistance of 100 colonies were scored. As expected, a high rate of antigenic conversion (88/100) was found while the not converted 12 clones proved not lysogenic. The same experiment under the same conditions was then performed with phage φ_{IV_1} (NTCC 4839). In this case, no antigenic convertants were obtained from the 100 colonies scored, while 68 of the 100 clones became lysogenic. Treated with phage φ_{IV_1} in an analogous way, the UP511 host-cells in which a reduced repressor production by the phage was observed, yielded no antigenic convertants, while the clones studied proved lysogenic in a ratio of 100/100, without showing a reduced repressor production against the homologous phage φ_{IV_1} or phage PE5.

Discussion

The converting phages of *Sh. flexneri* studied by us (except the known phage fII) form a group of closely related phages as shown by their electron microscopic morphology, plaque characteristics, host range, the results of neutralization tests, and last but not least by their formation of a homo-immune system. An important common feature of these phages is the lack of UV-inducibility.

Their antigenic conversion lends a practical and a not less significant theoretical importance to these phages. Due to the lack of more extensive knowledge on phage genetics, the question whether the gene for UDP-glycosyl transferase is of phage or of bacterial origin, in other words whether the converting phage is carrying the information or it only induces a bacterial gene, must be left open. The same question arises in connection with the toxin gene of *C. diphtheriae* [15].

In the conjugation experiments performed by MANSON, SIMMONS and PETROVSKAYA [16] using HfrC strain, the serotypes of *Sh. flexneri* studied changed for so-called Y₁ structures while losing their α -glucose side-chain carrying the type-specificity. As an explanation, a hypothetical bacterial T-locus was assumed to have been lost. However, the success of our developing various dilyso-genic converted derivatives may indicate that there are more than one "T-locus" viz., the *att* loci of these phages would not be identical. The possible occurrence of a tandem association of two phages — a phenomenon analogous to that occurring in mutants of the phage lambda — cannot be excluded [17]. Elucidation of this problem will require localization studies.

Our studies on phage immunity have prompted us to separate lysogenic conversion from the lysogenic condition. A highly reduced rate of repressor production was found in part of the convertants, with the degree of reduction depending on the phages and host-cells used. This observation raised the possibility that in these cases a gene exerting positive control on repressor production during the recombination process brought about by insertion of the prophage, might have become extincted. Some of our experiments offered convincing evidence that a lysogenizing phage does not inevitably lead to lysogenic conversion condition and that in these instances, also the reduction of repressor production fails to occur. During lysogeny, a fairly wide range of integration is known to take place from phage P1 where the prophage fails to integrate but remains cytoplasmic in a plasmid-like way [18, 19], to phage lambda where the event of reciprocal recombination between the prophage and the host-cell has been studied in detail [20]. One of the possibilities may be that it is during lysogenic conversion that such a level of integration occurs while simple lysogenization does not require such a degree of integration. The existence of several chromosites exhibiting perhaps preferential sequences

may be another possibility known to be the case in phage P2 [21]. For that matter, conversion functions (*fun*, *old*) have been found to be independent of the lysogenic condition also in phage P2.

Salmonella phage P22 offers a similar analogy, the more so as in the groups A, B and C of *Salmonella* it is capable of inducing the conversion of antigen O1 [22, 23] which is α -glucose and binds to the galactose (mannose-rhamnose) oligosaccharide at sites 1–4 [24]. The same phage is also capable of general as well as of special transduction [25, 26] and its preferential *att* locus is known to be localized between the loci *proA* and *proC* [27].

As shown by the experiments of LEMINOR [28], phages inducing antigen-conversion in *Salmonella* also form a uniform group on the basis of their electron-microscopic morphology (BRADLEY's group C) and all of them exhibit transduction properties.

The question raised as well as the hypotheses set forth may be answered only by further studies. Some practical implications are, however, worth mentioning. The overwhelming majority of the freshly isolated strains carrying the type antigens show that the strains lysogenic with these converting phages — like *Salmonella* — are widely spread in nature and there is no doubt as to the occurrence of a number of dilysogetic strains among them as it comes from the existence of serotype 2b and of some other serotypes. *Sh. flexneri* var. Y undoubtedly appears to be a "disinfected" strain. This statement is supported by the earlier observation [29] that this variant can be isolated with a frequency of 2.8% from cases of acute dysentery while with one of 19% from chronic carriers. No similar changes for var. X have been observed. It is also known from studies on the antigenic structure of *Sh. flexneri* that the question whether the antigenic scheme should be constructed on the basis of type-specific or of group antigens, has been the subject of much discussion. RAUSS [30] was the first to consider group antigens as the "back-bone" of the system of antigenic structure and he even set criteria for group antigens not only on the basis of their occurrence in several types but also by taking their stability into account. This concept is definitely supported by our present knowledge of the origin of antigens through lysogenic conversion.

It is also well-known that there are fine differences in the antigenic structure of the different type strains e.g. of strains 4a, 4b, "rio, and "rabaulensis" [31]. The antigen converted by our phage P90 and being in a,b—a,c relation with antigen IV₁ [3], may offer an explanation for such differences.

Another finding possibly of practical importance is presented by the history of the isolation of our phage PE5. This phage was isolated from strain 2a of *Sh. flexneri* where its converting capacity failed to become expressed. In other words, converting phages may be carried by strains in which the phenotypic expression of the phage is not detectable. A change in the anti-

genic structure may, however, by all means occur, making phenotypic expression possible. In this instance, the expression of antigen V in variant Y becomes possible after "disinfection" of antigen II has occurred (the same was observed with the var. Y strains studied) and a "type shift" scheme of 2a $\rightarrow$ var. Y $\rightarrow$ 5 may take place. All these occur without reinfection with other serotypes.

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EPIDEMIOLOGICAL EVALUATION OF THE EFFICIENCY OF ORAL VACCINATION AGAINST ENTEROPATHOGENIC *ESCHERICHIA COLI*

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Summary. Experimental vaccination of hospitalized infants was performed with the Rauss-type trivalent oral vaccine against enteropathogenic *Escherichia coli* infection. In the periods 1969—70 and 1970—71, each infant received for basic immunization 9 and 18 tablets, respectively. The maintenance dose was one tablet weekly throughout the period of hospitalization. The vaccine contained 0.5 mg each of freeze-dried Boivin-type extracts of *E. coli* O111 : B4, *E. coli* O55 : B5 and *E. coli* O86 : B7 per tablet. It dissolved readily in tea and had no noticeable side effect. A total of 627 vaccinees and 1040 unvaccinated controls were observed during the experimental period. Infantile enteritis associated with *E. coli* occurred in 28 vaccinees (incidence, 4.5%) and in 79 control individuals (incidence, 7.6%). The protective effect of the vaccine increased parallel with the age of the vaccinees, the rate of protection being 30% among new-born mature and premature babies, 55% among 1—2-month-old and 74% among 3-month-old infants. The mean protective effect was 40.8%. Vaccination had no influence on the clinical course of the disease, nor on the incidence of symptomless excretion. Among the unvaccinated controls, the incidence of infection increased parallel with the duration of hospitalization. The protective effect of vaccination was, however, demonstrable only during the first two months (65%) of hospitalization. In most cases the aetiological agent was *E. coli* O111 : B4. The symptomless excretors excreted several other *E. coli* serogroups as well. On the basis of the epidemiological evaluation, the vaccine appears to be a useful preventive measure against nosocomial epidemics. Since, however, delayed cases also occur, the present dosage schedule needs revision.

Infantile enteritis associated with *Escherichia coli* (IEc) is one of the most frequent and most important infant diseases [1]. Its importance as a iatrogenic damage is not negligible either. In Hungary, the number of annual hospital infections amounts to 1000—1200 cases (*i.e.* half of those reported [2]). The control of nosocomial epidemics is difficult, because once introduced into an infants' ward, they spread like prairie fire.

This is not surprising if we consider the high contagiousity of the disease, the great sensitivity of infants, the shortcomings of nursing hygiene, etc.

Thus a reduction of the infants' sensitivity by an appropriate vaccination would be decisive for the prevention and control of epidemics. Oral vaccination against IEC was first considered in the middle 'sixties. In East Germany, MOCHMANN and OCKLITZ [7—17] developed a potent vaccine and evaluated it serologically first in experimental animals, then in infants.

In Hungary, RAUSS and his team (Institute of Microbiology, University Medical School, Pécs) have investigated the problem of vaccination against

IEc. They have elaborated a combined vaccine, containing the enteropathogenic *E. coli* strains commonly occurring in this country. The tabletted oral vaccine produced a satisfactory rate of seroconversion in mouse-protection tests and later also in infants. Parallel to the rise of the serum antibody level, also copro-antibodies appeared. The methods of production and control of the vaccine, the proposed vaccination schedules and the results of serological evaluation have been described in a series of publication by RAUSS and his group [18–20].

This paper is a report of the epidemiological evaluation of the effectiveness of the Rauss-type vaccine. The trial was performed in the Department of Paediatrics, University Medical School, Szeged.

Materials and methods

Vaccine. The tabletted oral vaccine used in the trial was prepared by RAUSS and his team. Each tablet contained a freeze-dried Boivin-type extract of 50×10^9 cells (0.5 mg dry substance) from *E. coli* O111 : B4, *E. coli* O55 : B5 and *E. coli* O86 : B7. The tablets dissolved readily in tea. No untoward side effect occurred throughout. In the first vaccination experiment the control subjects were given placebo tablets.

Vaccination schedule. Treatment consisted of basic immunization and maintenance. According to the instructions of RAUSS, in the first experimental period (1969–70) 3 tablets were given on 3 occasions at 5-day intervals for basic immunization, viz. a total of 9 tablets within 10 days, corresponding to 3×9.0 mg of antigen. For maintenance, one tablet was given every week as long as the infant was kept in the hospital.

Organization and documentation of the vaccinations. Group vaccinations including appropriate controls were conducted in the Department of Paediatrics of Szeged University from August 1st, 1969 to December 31st, 1971. The infant bed capacity of the Department is 150, including 35 beds in the premature ward. The babies received vaccine or placebo treatment in the sequence of admission. After the first year of the experimental period, the placebo was omitted.

The Department supplied a form for each baby, informing apart from general data (disease, body weight, period of nursing, etc.) about the basic disorder, laboratory results and, if the diagnosis was IEc, on the course and other characteristics of the disease. The statistical data presented here were compiled on the basis of these forms.

Evaluation of vaccination history. During the 2.5-year period, forms were supplied for a total of 2068 babies, out of which 627 (30.3%) had been vaccinated, 1040 (50.3%) had not been vaccinated, and 401 (19.4%) could not be evaluated in lack of information.

The baby was regarded as vaccinated if he had received a complete basic immunization and subsequent weekly maintenance treatment for the period of hospitalization if it was not interrupted for more than 14 days. Babies meeting these requirements formed the vaccinated group.

The infant was regarded as unvaccinated if it had received no vaccination treatment whatever. In the first year of the experiment, placebo tablets were given instead of the oral vaccine. Babies belonging to this category formed the control group.

The baby was excluded from evaluation, if he had

- (1) been admitted with IEc, or in the period of latency, developing the disease within 5 days after admission (28 cases);
- (2) been discharged or died within 5 days after admission, and thus was not available for observation (181 cases);
- (3) had no complete basic immunization (94 cases);
- (4) developed IEc in the course of, or within 3 days after the completion of, basic immunization, that is, the immune state could not be judged (15 cases);
- (5) excreted an agent of a serological type not included into the vaccine (14 cases), or no pathogenic agent was demonstrable in the faeces, despite an existing diarrhoea (6 cases).

Since most babies excluded from evaluation represented vaccinees, the number of control subjects was much higher at evaluation.

Results

Prior to epidemiological evaluation, it had to be ascertained whether the randomization of the babies corresponded with the condition of the trial, *viz.* whether the distribution of the most important factors influencing infantile susceptibility was uniform in the experimental and the control group. The protective effect was assessed from the difference in morbidity rate between the two groups. Some characteristics of IEc were compared in vaccinated and unvaccinated subjects, correlation was sought between the number of vaccinations and the course of the disease, laboratory results, etc.

I. Study of randomization

1. The seasonal distribution of admissions was fairly even in both groups. In fact, no difference was found in this respect (Table I).

Table I
Distribution of vaccinated and unvaccinated infants according to quarterly admission

Months	No. of admitted infants			Per cent of admitted infants		
	Vaccinated	Un-vaccinated	Total	Vaccinated	Un-vaccinated	Total
January—March	152	220	372	24.2	21.1	22.3
April—June	141	268	409	22.5	25.8	24.5
July—September	150	257	407	23.9	24.7	24.4
October—December	184	295	479	29.4	28.4	28.8
Total	627	1040	1667	100.0	100.0	100.0

2. The body weight distribution of the infants was at variance chiefly at the low body weight level. On the average, one third of the infants had been born with less than 2500 g body weight, and one quarter had a body weight of 2500—4000 g. The number of low weight babies was somewhat higher in the experimental than in the control group, whereas in the control group babies of the 2500—4000 g body weight range were somewhat more numerous, but these slight differences could be equalized. The higher body weight groups were roughly uniform among vaccinees and controls (Table II).

3. The age distribution of the infants was also roughly uniform in the two groups. Half of the babies were newborn (0-month-old); nearly all low weight babies and one third of the mature newborns belonged to this age category. The other half of the subjects was older than one month. It is known that IEc-infections threaten chiefly the youngest infants (Table III).

Table II
Body weight of infants at admission

Body weight, g	No. of admitted infants			Per cent of admitted infants		
	Vaccinated	Un-vaccinated	Total	Vaccinated	Un-vaccinated	Total
Less than 2500	223	304	527	35.6	29.2	31.6
2501—4000	140	284	424	22.3	27.3	25.4
4001—7000	140	249	389	22.3	23.9	23.4
More than 7001	124	203	327	19.8	19.6	19.6
Total	627	1040	1667	100.0	100.0	100.0

Table III
Distribution of infants according to age at admission

Age (months)	Number of infants			Per cent		
	Vaccinated	Un-vaccinated	Total	Vaccinated	Un-vaccinated	Total
a. Low-weight babies						
0—month-old	222	293	515	35.4	28.2	30.9
1—2-month-old	1	11	12	0.2	1.0	0.7
Total	223	304	527	35.6	29.2	31.6
b. Mature babies						
0-month-old	104	214	318	16.6	20.6	19.1
1—2-month-old	71	118	189	11.3	11.3	11.3
Above 3 months	229	404	633	36.5	38.9	38.0
Total	404	736	1140	64.4	70.8	68.4
Total	627	1040	1667	100.0	100.0	100.0

4. Distribution according to basic disease was roughly uniform in the two groups. Thirty per cent had been hospitalized for low weight at birth, 20% for respiratory disease (pneumonia, etc.), and 14% had a congenital abnormality (Table IV).

5. Distribution according to the clinical course of the basic disease was identical in the two groups. Sixty-seven infants (4%) died from a disease other than IEc (Table V).

6. The duration of hospitalization is an important point in respect of exposure. Half of the infants were hospitalized for a short term, and were discharged within 3 weeks. Babies hospitalized for less than 5 days were ex-

Table IV*Clinical forms of basic disease in the infant groups studied*

Basic disease	Number of infants			Per cent		
	Vaccinated	Un-vaccinated	Total	Vaccinated	Un-vaccinated	Total
Premature birth	203	271	474	32.4	26.1	28.4
Enteric disease	19	31	50	3.0	3.0	3.0
Respiratory disease	133	196	329	21.2	18.8	19.8
Cutaneous disease	12	25	37	1.9	2.4	2.2
Congenital abnormality	76	151	227	12.1	14.5	13.6
Congenital heart defect	25	52	77	4.0	5.0	4.6
Congenital neurological disease	22	35	57	3.5	3.4	3.4
Atrophy	6	5	11	1.0	0.5	0.7
Other disease	131	274	405	20.9	26.3	24.3
Total	627	1040	1667	100.0	100.0	100.0

Table V*Clinical course of basic disease*

Clinical course	Number of infants			Per cent		
	Vaccinated	Un-vaccinated	Total	Vaccinated	Un-vaccinated	Total
Mild	152	216	368	24.2	20.8	22.1
Medium severe	311	494	805	49.6	47.5	48.3
Severe	126	256	382	20.1	24.6	22.9
Lethal	18	49	67	2.9	4.7	4.0
Unknown	20	25	45	3.2	2.4	2.7
Total	627	1040	1667	100.0	100.0	100.0

cluded from the evaluation, as already noted in the foregoing. The distribution of hospitalization periods was roughly uniform in the two groups (Table VI).

Scrutiny of the vaccinated and control groups from different points of view has thus confirmed that randomization was correct, because distribution according to different criteria displayed no notable differences, allowing a realistic evaluation.

Table VI
Duration of hospitalization

Number of days in hospital	Number of infants			Per cent		
	Vaccinated	Un- vaccinated	Total	Vaccinated	Un- vaccinated	Total
6— 9	148	283	431	23.6	27.2	25.9
10—19	185	321	507	29.5	31.0	30.4
20—29	112	173	285	17.9	16.6	17.1
30—59	118	174	292	18.8	16.7	17.5
More than 60	64	88	152	10.2	8.5	9.1
Total	627	1040	1667	100.0	100.0	100.0

II. Epidemiological evaluation of the vaccinations

Efficiency of the vaccine was assessed in the usual manner, by comparison of morbidity rates between vaccinees and unvaccinated controls. Twenty-eight babies (4.5%) developed the disease in the vaccinated group, and 79 (7.6%) in the control group; the total number of diseased babies was 107, corresponding to 6.4% of the babies under observation.

The rate of protection amounted to 40.8%, *viz.* vaccination effected the decrease of the morbidity rate by 41% as compared to the controls. This result in itself was not favourable, but grouping of the results according to age and to the period of hospitalization puts it in an entirely different light.

1. Relationship of protective effect with age. Morbidity decreased, whereas the protective effect increased with age. 0-month-old babies, whether mature or premature, responded weakly to immunization; in this age group, the rate of protection was only 30%. At the age of 1—2 months, the protection rate rose to 54% and at 3 months, or above, to 74%, the average was 66%, twice as high as in the 0-month category. This rate can already be regarded as a good medium degree of protection (Table VII).

2. The morbidity rate tended to increase with the duration of hospitalization, because long-term hospitalization increases the time of exposure and along with it the risk of infection. The total incidence of IEc among vaccinated and unvaccinated babies treated for periods of up to one month, up to two months and three months or more was 2.5, 14.4 and 23.0%, respectively. Morbidity rates differed notably, in favour of the vaccinees, during the first two months, as shown by a protection rate of 65% for this period. After the third month, the morbidity rates did no longer differ, indicating that the vaccination had lost its efficiency (Table VIII).

Table VII

Incidence of infantile enteritis associated with E. coli according to age and maturity of infants

Age (months)	Infants vaccinated			Infants not vaccinated			Protective effect, per cent
	Total No.	Diseased		Total No.	Diseased		
		No.	per cent		No.	per cent	
Prematurely born babies	223	17	7.6	304	33	10.9	30.3
Mature babies							
0-month-old	104	5	4.8	214	15	7.0	31.4
1—2-month-old	71	3	4.2	118	11	9.3	54.8
Above 3 months	229	3	1.3	404	20	5.0	74.0
Total	627	28	4.5	1040	79	7.6	40.8

Table VIII

Distribution of infantile enteritis associated with E. coli according to duration of hospitalization

Number of days in hospital	Infants vaccinated			Infants not vaccinated			Protective effect per cent
	Total No.	Diseased		Total No.	Diseased		
		No.	per cent		No.	per cent	
6— 9	148	—	—	283	3	1.1	.
10—12	185	1	0.5	322	9	2.8	.
20—29	112	4	3.6	173	13	7.5	.
Total	445	5	1.1	778	25	3.2	65.7
30—59	118	8	6.8	174	34	19.5	65.1
More than 60	64	15	23.4	88	20	22.7	—
Total	627	28	4.5	1040	79	7.6	40.8

3. Analysis of the interval between admission and the onset of IEc has led to similar conclusions. Half of the vaccinated patients developed the disease beyond 30 days after admission, because vaccination effected a delay of onset. No such temporal shifts were observed among the unvaccinated subjects (Table IX).

4. Vaccination had no influence on the clinical course of EIEc: the distribution of mild, medium severe, and severe cases was uniform in the experimental and the control group. The mortality rates did not significantly differ,

nor were they conclusive in view of the low number of fatal cases. Vaccination had no influence on symptomless excretion: the number of excretors was almost identical in the experimental and the control group (Table X).

Table IX

Time interval between admission and onset of disease

Number of days between admission and onset	Infants developing IEc during hospitalization			
	Vaccinated		Unvaccinated	
	No.	per cent	No.	per cent
5—9	—	—	21	26.6
10—14	3	10.7	16	20.2
15—30	10	35.7	23	29.1
More than 30	15	53.6	19	24.1
Total	28	100.0	79	100.0

Table X

Clinical course of infantile enteritis associated with E. coli

Clinical course (stay in hospital)	Vaccinated infants		Unvaccinated infants	
	No.	per cent	No.	per cent
Mild (1—4 days)	9	32.1	26	32.9
Medium severe (5—9 days)	12	42.9	30	38.0
Severe (beyond 9 days)	6	21.4	16	20.2
Lethal	1	3.6	7	8.9
Total number of patients	28	100.0	79	100.0
Symptomless excretors	22	3.5	35	3.4

5. The incidence of IEc tended to increase with the degree of severity of the clinical course of the basic disease. Gravely ill babies showed an increased susceptibility to infection, whether or not they had been vaccinated (Table XI).

6. Results of bacteriological examinations were similar in the experimental and the control group. In all but three cases of IEc, the aetiological agent was *E. coli* O111 : B4 (93.7%). *E. coli* O55 : B5 was responsible for the condition in 3 cases and no *E. coli* O86 : B7 occurred in the faecal samples. This distribution differed slightly among the symptomless excretors (Table XII).

Table XI

Incidence of infantile enteritis according to clinical course of basic disease

Clinical course of basic disease	Vaccinated infants			Unvaccinated infants		
	Total	Diseased		Total	Diseased	
		No.	per cent		No.	per cent
Mild	152	3	2.0	216	8	3.7
Medium severe	311	13	4.2	494	36	7.3
Severe	126	7	5.6	256	27	10.5
Lethal	18	5	27.8	49	8	16.3
Unknown	20	—	—	25	—	—
Total	627	28	4.5	1040	79	7.6

Table XII

Laboratory results

<i>E. coli</i> serogroup	Positive isolations					
	Total		From diseased subjects		From excretors	
	No.	per cent	Vaccinated	Unvaccinated	Vaccinated	Unvaccinated
O111 : B4	139	84.8	27	77	11	24
O55 : B5	10	6.1	1	2	6	1
O86 : B7	15	9.1	—	—	5	10
Total	164	100.0	28	79	22	35

III. Incidence of IEc among vaccinated and control subjects

1. Forty-five per cent of the vaccinees received only basic immunization, owing to the short period of hospitalization. Out of them 85 babies (13%) were given 3×3 tablets at 5-day intervals, 201 babies (from the second year on) 3 tablets daily for 6 days (32%). Only four infants (1.4%) of the two groups developed IEc.

More than half of the babies received maintenance doses. According to the period of hospitalization, 29% received 1–2 maintenance doses and 25% received 3 or more doses. Paradoxically, the incidence of IEc was 3% among the former and 12% among the latter, *viz.* more babies contracted the disease among those who had received a greater number of maintenance doses. The greater number of maintenance doses meant of course a prolonged stay in the hospital and vaccination obviously cannot prevent the dangers of a long-term exposure (Table XIII).

Table XIII*Relationship between number of vaccinations and disease*

No. of vaccinations	Infants vaccinated		Infants diseased	
	No.	per cent	No.	per cent
1. Basic immunization only				
a. 3 × 3 tablets	85	13.6	1	1.2
b. 6 × 3 tablets	201	32.0	3	1.5
Total	286	45.6	4	1.4
2. Basic immunization + maintenance				
a. 1—2 doses	181	28.9	5	2.8
b. 3 or more doses	160	25.5	19	11.9
Total	341	54.4	24	7.0
Total	627	100.0	28	4.5

2. The analysis of the time interval between the last vaccination and the onset of IEc was not conclusive. IEc occurred in most cases within 3 days after the conclusion of maintenance vaccination. Onsets within 3 days after basic immunization had *ab ovo* been excluded from evaluation, for reasons outlined in the methodological part of this paper. Onsets occurring more than 14 days after the last vaccination have also been excluded, in view of the long interruption (Table XIV).

Table XIV*Interval between last immunization and onset of disease*

No. of days	No. of vaccinations				Total
	Basic immunization only	No. of maintenance doses			
		1—2	3—5	>6	
1—2	—	3	7	7	17
4—7	2	1	2	2	7
8—14	2	1	—	1	4
Total	4	5	9	10	28

Discussion

The results of oral vaccination of large, well-randomized groups of infants can be summarized as follows.

1. The average reduction of the morbidity rate of IEc was 40% among the vaccinees, as compared to the unvaccinated controls.

2. The vaccination protected against the infection mainly the babies older than 1 month. The protective effect increased with age: the rate of protection was only 30% among the newborns, 54% at 1–2 months of age, and 74% at 3 months of age or more. Thus the results of vaccination in a given infant ward depend on the proportion of newborns; the less numerous the latter, the more favourable the result.

3. Vaccination was effective only if hospitalization had lasted no longer than 2 months. From the third month, protection declined, as judged from the equal morbidity rate in the experimental and control groups. Maintenance doses did not notably prolong the duration of immunity. If the infant is hospitalized for a longer period, basic immunization should preferably be repeated, or performed *ab ovo* with a dose high enough to render repetition unnecessary. Recent investigations of RAUSS [21] have shown that the result can be improved by increasing the dosage.

4. The same conclusion was drawn from the analysis of the time interval between admission and onset of illness, and from the effect of the number of maintenance doses on the duration (degree) of protection. Basic immunization was found to confer the substantial part of immunity and protection declined parallel to the time elapsing after basic immunization.

5. All but three nosocomial infections were caused by *E. coli* O111 : B4. Organisms corresponding to the other two components of the trivalent vaccine had no notable role in the disease, but they occurred in several faecal samples from symptomless excretors.

6. Oral vaccination had no influence on the clinical course of IEc, nor on the numerical relations of symptomless excretors.

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EFFECT OF CHLORAMPHENICOL ON PHOSPHOLIPID SYNTHESIS IN SENSITIVE STAPHYLOCOCCUS AUREUS STRAINS

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Summary. Chloramphenicol has been found to alter the phospholipid synthesis in *Staphylococcus aureus* at a concentration of 3 $\mu\text{g/ml}$, which did not inhibit bacterial multiplication. The lysyl-phosphatidyl-glycerol content was reduced from 20% to 4%, the quantity of phosphatidyl-glycerol increased from 51% to 66%, while cardiolipin content was unaffected. The fatty acid content of phospholipids also changed, the quantity of straight chain saturated fatty acids decreased, that of branched chain fatty acids increased.

Chloramphenicol has a specific inhibitory effect on ^{32}P incorporation into lysyl-phosphatidyl-glycerol when added to a culture in the logarithmic phase of growth. The inhibitory effect of chloramphenicol on lysyl-phosphatidyl-glycerol synthesis can be demonstrated also in cultures which are in the lag phase.

Chloramphenicol inhibits the protein synthesis of bacteria. There are different assumptions and experimental data concerning the mechanism of this effect. According to DAS *et al.* [1] and WEBER and DEMOSS [2] the drug interferes with one of the steps of polypeptide synthesis occurring in the ribosomes. According to VÁZQUEZ and MONRO [3] and WOLFE and HAHN [4], the only specific effect of chloramphenicol is the inhibition of binding to ribosomes of code specific tRNA. While investigating the kinetics of peptide release from transfer ribonucleic acids under the effect of puromycin, WEBER and DEMOSS [5] demonstrated that chloramphenicol did not affect the peptidyl transferase reaction. They inferred from this that chloramphenicol exerted its effect on another phase of protein synthesis prior to the reaction occurring on the surface of ribosomes.

The effect of chloramphenicol on lipid synthesis has not been investigated. Our examinations were based on the assumption that chloramphenicol had a selective inhibitory effect. On the one hand, the synthesis of different proteins may have a different sensitivity to the action of chloramphenicol and, on the other, chloramphenicol may have a direct inhibitory action on enzymatic processes. It may be membrane coupled for a time and thus inhibit the membrane-bound enzymes in the course of penetration.

The phospholipid metabolism of bacteria cannot be separated from membrane function, because more than 90% of the phospholipids are localized in membranes. Therefore, we have attempted to study the mechanism of the

chloramphenicol effect by observing the changes occurring in phospholipid synthesis.

Materials and methods

Investigated strains: *Staphylococcus aureus* 100 and 80/81 coagulase positive strains.

Culturing. A 1% Difco Casiton culture medium was used supplemented with 1% sterile glucose solution before inoculation; 150 ml culture fluid was introduced into 500 ml Erlenmeyer flasks. Inoculation was carried out with 1 ml of an 18-hour fluid culture, incubation was done under shaking at 37°C for 18 hours. Antibiotics were added in 3 µg/ml concentration just before inoculation. Bacterial growth was estimated by counting the colony forming units and determining the dry material content.

Lipid extraction. The bacteria cultured for 18 hours were washed twice with physiological saline, then the lipids were extracted as described in [6]. Extraction was repeated twice, then the extracts were collected and dried in inert atmosphere. The total lipids obtained were purified on a Sephadex-G-25 column according to the method of WELLS and DITMER [7].

Lipid fraction, thin-layer chromatography of phospholipids, and lipid phosphorus determination were carried out as described earlier [8].

Examination of ^{32}P incorporation. (1) One µCi $\text{Na}_2\text{H}^{32}\text{PO}_4$ per ml was added to the culture in the phase of logarithmic growth; samples were taken 0, 1, 2, 3 hours later and the radioactivity incorporated into phospholipids was measured. Chloramphenicol was introduced into the culture just before isotope administration.

(2) A 3000 ml amount of a bacterial suspension cultured for 18 hours in a medium free of antibiotics was centrifuged, washed twice with saline, and stored at 4°C for 18 hours. Subsequently, the bacteria were centrifuged and suspended in 1000 ml phosphate-free Difco Casiton culture medium at 37°C. The pH was adjusted by Tris buffer. The culture medium contained 1 µCi/ml ^{32}P .

^{32}P incorporation was stopped by the introduction of 4 N hydrochloric acid and ice; subsequent centrifugation and washing were carried out at 4°C.

The radioactivity of phospholipids was measured directly on the thin-layer chromatogram with an end window Geiger-Müller tube, that of the eluted lipids was measured with a Packard scintillation spectrometer.

Results

In preliminary experiments chloramphenicol in an initial concentration of 3 µg/ml had no significant effect on the number of colony forming units or dry material content during an 18-hour incubation period. Not even the extractable total lipid content of *Staph. aureus* displayed a significant change. However, there was a significant change in the quantity of phospholipids present in *Staph. aureus* in the presence of chloramphenicol at a concentration which did not affect bacterial growth.

As demonstrated in Table I the lysyl-phosphatidyl-glycerol (LPG) content of *Staph. aureus* was reduced from the 20% control value to 4% when grown on a medium with chloramphenicol (3 µg/ml). Simultaneously, the phosphatidyl-glycerol (PG) content increased from 51% to 66% while the quantity of cardiolipin (DPG) was unchanged. The quantity of phosphatidic acid (PA) could not be evaluated as it only amounted to 1–2% of the total phospholipids. All this suggested that not only the quantitative relations of phospholipids but also their synthesis underwent a change under the effect of chloramphenicol.

Table I

*Effect of chloramphenicol (3 $\mu\text{g/ml}$) on phospholipid synthesis in Staph. aureus**

	LPG	PG	PA	DPG
Control	20	51	2	26
Chloramphenicol (3 $\mu\text{g/ml}$)	4	66	1	28

* On the basis of phospholipid P content, in per cent of total phospholipid content; average of 3 determinations

Table II presents the per cent distribution of the main fatty acids in the phospholipids of *Staph. aureus*. In the presence of chloramphenicol (3 $\mu\text{g/ml}$) the fatty acid content of all the three phospholipids had changed. The change was more expressed in the case of PG and cardiolipin than in the case of LPG. Characteristic was the decrease of the straight chain saturated fatty acids and the increase of branched-chain fatty acids.

³²P incorporation into PG and LPG. Fig. 1 demonstrates ³²P incorporation with and without chloramphenicol. ³²P was added to the culture in the phase of logarithmic growth.

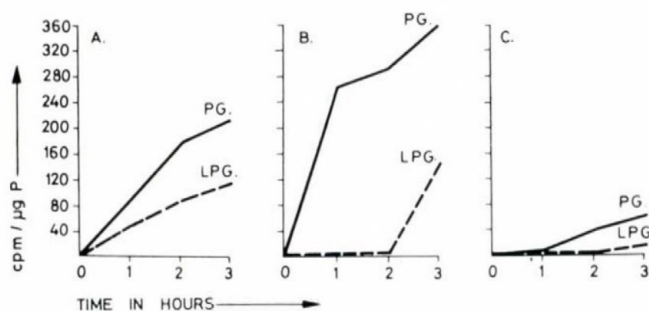


Fig. 1. ³²P incorporation into *Staph. aureus* PG and LPG, with the isotope administered in the logarithmic phase. A = control; B = 20 μg chloramphenicol per ml; C = 50 μg chloramphenicol per ml. Specific activity is related to the phosphorus content of phospholipids. Activity was measured by a Packard scintillation spectrometer

Chloramphenicol at 20 $\mu\text{g/ml}$ completely inhibited ³²P incorporation into LPG during the first 2 hours of incubation. Following the second hour, incorporation increased rapidly. Chloramphenicol at this concentration did not decrease ³²P incorporation into PG but even increased it.

Chloramphenicol at 50 $\mu\text{g/ml}$ completely inhibited ³²P incorporation into LPG during 3 hours incubation while PG showed a slow increase of activity after the first hour of incubation.

Table II
Main fatty acids of *Staph. aureus* in per cent
of total fatty acid content

Fatty acids	Control			Chloramphenicol (3 µg/ml)		
	LPG	PG	DPG	LPG	PG	DPG
C ₁₄	12.6	1.6	4.6	7.8	0.4	4.6
aiC ₁₅	33.8	42.6	41.4	39.4	55.5	53.9
C ₁₆	26.7	2.1	10.1	15.6	1.7	9.2
iC ₁₇	7.0	3.4	6.9	10.5	15.4	13.1
C ₁₈	11.2	17.7	13.3	7.8	8.2	7.8
C ₂₀	8.4	23.3	10.5	7.0	8.0	1.8

The LPG synthesis-inhibiting effect of chloramphenicol could be also observed in bacterial cultures previously "fasting" for 18 hours, which failed to multiply actively under the experimental conditions.

Fig. 2 demonstrates the ³²P incorporation of a resting *Staph. aureus* culture on a medium without and with 100 µg chloramphenicol per ml. At

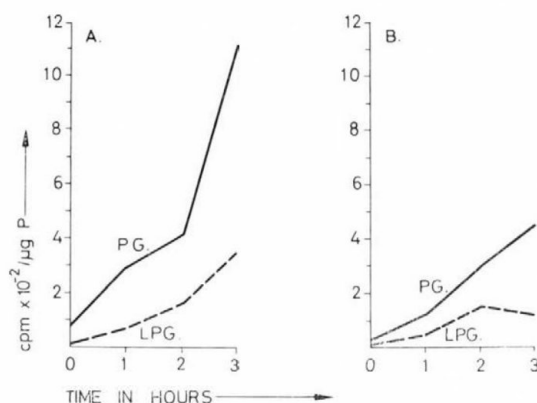


Fig. 2. ³²P incorporation in lag-phase of *Staph. aureus*. A = control; B = 100 µg chloramphenicol per ml. Activity was measured by a Packard scintillation spectrometer

this concentration chloramphenicol strongly inhibited ³²P incorporation into the PG and the LPG fractions during the first hour of incubation. Subsequently, the activity of PG gradually increased while that of LPG decreased.

Discussion

The effect of antibiotics on lipid synthesis is scarcely known. According to HUGO and STRETTON [9] an increase in the lipid content of *Staph. aureus* is accompanied by an increase in the resistance to penicillin. As shown by BERMINGHAM *et al.* [10] streptomycin has a specific inhibitory effect on the

synthesis of cyclic depsi-peptides of *Serratia marcescens* at a concentration which has no effect on bacterial growth. The total lipid-phosphorus content displayed a twofold increase. According to our own data, the phospholipid content of both methicillin sensitive and resistant *Staph. aureus* strains changes under the effect of methicillin while the quantity of cardiolipin significantly increases in sensitive as well as in resistant strains [11].

The present results agree well with the data of GOULD and LENNARZ [12] and SHORT and WHITE [13] in that among the phospholipids of *Staph. aureus* the metabolism of PG is more rapid than that of LPG. According to SHORT and WHITE [13] the LPG content of *Staph. aureus* does not increase in the course of reproduction, while during the resting phase the PG content decreases and the cardiolipin content increases.

It may be stated on the basis of the present results that LPG synthesis in *Staph. aureus* is inhibited by chloramphenicol at a concentration which has no effect on bacterial growth. The PG content of bacteria increases in the presence of chloramphenicol during an 18-hour incubation period. Inhibition of LPG synthesis occurred at a chloramphenicol concentration which did not interfere with PG synthesis and even increased phospholipid synthesis or caused an accumulation of phospholipids.

According to GIESBRECHT and RUSKA [14], chloramphenicol at 5 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ concentration induces an expressed thickening of the cell wall of *Staph. aureus*. The cytoplasm mass is reduced as compared to the mass of the cell wall. Chloramphenicol treatment inhibited cytoplasmic protein synthesis while the cell wall synthesis increased; the result was a change of the proportion of cytoplasm and cell wall. Chloramphenicol treatment had an effect on the synthesis of bacterium membranes, too: an increase of mesosomes was observed in chloramphenicol-treated bacteria.

KING *et al.* [15] investigated the ultrastructural changes of mitochondria of HeLa and L cells in the course of chloramphenicol treatment. They demonstrated that the mitochondrion membrane structure was altered under the effect of chloramphenicol: the inner membrane degenerated. At the same time, the activity of cytochrome c reductase, succinic dehydrogenase and cytochrome b displayed a fourfold decrease.

SMITH-JOHANNSEN and GIBBS [16] examined the changes of chloroplasts and mitochondria of *Ochromonas danica* (*Chrysophyta* alga) and found that chloramphenicol severely damaged the membrane structure of chloroplasts and mitochondria. These morphological changes were explained by the specific inhibitory action of chloramphenicol on protein synthesis of mitochondrial ribosomes.

If we compare our own data to the above-mentioned experimental results the question arises whether the change observed was due to an inhibi-

tion of protein synthesis of mitochondrial ribosome. Chloramphenicol causes the degeneration of membranes carrying the energy-producing enzymes and might thus inhibit the function of these enzymes. It may be assumed that the destruction of the membrane structure is due to an inhibition of lipid synthesis, resulting in the disturbance of lipid metabolism.

In our experiments, chloramphenicol inhibited LPG synthesis at a concentration which had effect neither on the synthesis of other phospholipids nor on bacterial growth. The disturbed lipid synthesis causes changes in the membrane structure and this results in a changed metabolism of the bacteria. The effect on membranes and membrane bound enzymes might have a primary importance among the effects of chloramphenicol.

The inhibitory mechanism of chloramphenicol on lipid synthesis is not clear. GORDON *et al.* [17] investigated the effect of chloramphenicol and erythromycin on enzymes and lipid synthesis in wild and petite yeast mutants. They found that in wild strains chloramphenicol inhibited lipid synthesis to 50% under aerobic conditions while no significant effect on lipid synthesis of petite cells could be demonstrated under similar conditions. The authors could not explain the mechanism of this effect and supposed that chloramphenicol was bound to mitochondrial ribosomes and interfered with autocatalytic functions. Erythromycin had no inhibitory effect on lipid synthesis. It did not seem probable that the primary cause of the disturbed lipid metabolism was the inhibition of mitochondrial protein synthesis and the uncoupling of oxidative phosphorylation because an inhibitory effect was exerted by D-chloramphenicol but not by L-chloramphenicol. Further examinations are necessary to clarify the effect of chloramphenicol on lipid synthesis and to establish the mechanism of its action.

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INHIBITOR-SENSITIVE AND INHIBITOR-RESISTANT INFLUENZA A2 VIRUS POPULATIONS

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Summary. In the influenza A strains isolated from the 1957 pandemic, the inhibitor-sensitive (IS), in those of the 1959 epidemic, the inhibitor-resistant (IR) particles were predominant; for all the subsequent influenza A strains isolated so far the IR/IS ratio fluctuated around 1 : 1000. Pure IS and IR lines while undergone serial passages in embryonated eggs retained their IS and IR character, respectively. However, the IS line yielded an IR mutant at an approximately constant ratio in each passage. An artificial mixture containing 100 HA units of IS virus per each HA unit of IR virus was subjected to serial passages in embryonated eggs and, parallelly, in mice. The IR/IS ratio decreased in the egg passages while it increased in the mouse passages. Passages in embryonated eggs in the presence of normal sera from different animal species resulted in IR lines consisting of virions of different inhibitor resistance. The IS and IR lines obtained from the same strain showed no difference in their haemagglutinin antigen. The so-called A2 strains are different from the earlier influenza A strains in the mechanism of the adsorption to, and elution from, erythrocytes, these processes being influenced in the case of A2 strains, besides the mucoprotein receptors, by receptors of protein nature. The virus does not elute from these receptors by its neuraminidase enzyme. The properties of the IR and IS lines are summarized and discussed.

The antigenic structure of the human influenza A2 virus is H2N2 or H3N2 [1], *i.e.*, N2 neuraminidase is common to all A2 strains, whereas the strains isolated in the period from 1957 to 1968 and those having prevailed since 1968 (Hong Kong strains) are possessing different haemagglutinins.

Besides its distinctive antigenic structure, the A2 subtype has another peculiarity, *viz.*, the virion reacts, in addition to the mucoprotein inhibitor (α inhibitor) and receptor, with a serum inhibitor (γ inhibitor) and erythrocyte receptor, both of protein nature [2–6]. Consequently, the virion–inhibitor and virion–receptor reactions are in several respects different from the corresponding reactions of the earlier subtypes of influenza virus. The most pronounced difference is that neither the γ inhibitor nor the protein receptor is destroyed enzymatically by the virion. Consequently, the virion is neutralized by the inhibitor [2, 4, 5] and the virus adsorption on the erythrocyte is relatively stable [6, 7].

It is a further characteristic of the A2 subtype that the population of its strains is heterogeneous, consisting of IS and IR virions, *i.e.*, virions sensitive and resistant, respectively, to the γ inhibitor and the protein receptor [7, 8].

Considering the close chemical relationship between the serum inhibitors and the erythrocyte receptors of influenza virus strains, attempts have been made to reveal further characteristics of the IS and IR virions and, on the basis of these, of the mechanism of virion—receptor and virion—inhibitor reaction.

Materials and methods

Virus strains. Influenza A PR8/34 (HON1), Singapore/1/57 (H2N2) IR, Hong Kong/1/68 (H3N2) IS and IR, and IS and IR variants of influenza A (N2H2) virus strains isolated in Hungary in the period from 1957 to 1964.

Haemagglutination (HA) and HA-inhibition (HI) reactions were carried out according to the Microtitrator system [9].

The serum absorption test has been described earlier [10].

Results

Selection experiments. The methodological basis of these experiments has been the recognition that the IS virions of the A2 virus are neutralized by the protein inhibitor. The existence of IS and IR virions was demonstrated by CHOPPIN and TAMM [7, 8] in neutralization tests carried out with non-immune horse serum. We examined strains isolated in Hungary as well as prototype strains in a similar manner. Normal horse serum was heated to 60°C and cooled, then added to equal volumes of serial tenfold virus dilutions from 10^0 to 10^{-4} . To each of the higher virus dilutions (10^{-6} , 10^{-7} and 10^{-8}) an equal volume of PBS was added instead of horse serum. The mixtures were kept at +4°C for an hour and then inoculated into the allantoic cavity of embryonated eggs. The eggs inoculated with the virus—serum mixtures yielded IR virus, whereas those inoculated with highly diluted virus without serum yielded IS virus. The virus yields were estimated by the HA test. The selection of IS and IR variants from the Hong Kong/1/68 strains is shown in Table I.

In the presence of horse serum, the Hong Kong virus multiplied up to the 10^{-3} dilution and the recovered virus proved to be IR. An IR line was developed from the positive allantoic fluid (enframed in Table I) by serial passages with limit dilutions in the absence of horse serum.

The selection experiments carried out with Hungarian strains isolated in 1957 and 1959 are presented in Table II.

All the strains isolated from the 1957 pandemic proved to be IS, the IR virus yield being as little as $10^{0.3}$ to $10^{4.5}$ infectious units/ml. Some of the strains isolated from the 1959 epidemic, on the other hand, were less sensitive, or even resistant, to the inhibitor, *i.e.*, yielded populations in which IR virions were predominant. The titre of these strains was approximately equal in the

presence and in the absence of horse serum, therefore their IS component could not be determined quantitatively.

All the strains isolated from later epidemics in Hungary, including H2N2 and H3N2 strains, proved to be IS. Their IR virus yield did not exceed 10^3 infectious units/ml.

Table I

*Selection of IR and IS lines
from the influenza A2(Hong Kong)1/68 strain*

Selection of IR line with undiluted horse serum					Selection of IS line at limit dilutions		
HA titres of allantoic fluids from eggs inoculated with							
10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸
405	243	243	—	—	729 729 405 405	405 729 — 405	— — — —
243	135	405	—	—			
405	—	243	—	—			
405	243	—	405	—			

Table II

*Inhibitor sensitivity of influenza A2 virus strains
isolated in Hungary in 1957 and 1959*

Strain No.	Year of isolation	Degree of sensitivity	Log infectious units per 0.1 ml allantoic fluid	
			IS	IR
23, 24, 26, 28, 31, 33 and 37	1957	+++	6.4—7.5	0.3—2.5
25, 27, 30, 34, 35 and 36	1957	++	6.5—7.4	2.0—4.5
1, 8, 9, 13	1959	++++	5.8—6.9	0.2—1.3
10	1959	++	6.0	3.2
3, 5, 6, 7, 11	1959	+	5.3—6.9	4.0—6.9
2, 4, 12	1959	—	?	6.5—6.8

Crosses mean the range of HA titre of a standard inhibitor preparation as tested with the corresponding strain.

Ranges: +++ 1 : 20 000
 ++ 1 : 2 000—1 : 10 000
 + 1 : 100—1 : 1 000
 — below 1 : 100

Purity and stability of the IR and IS lines were checked by subjecting them to further passages in the presence of horse serum and at limit dilutions, respectively. The IR line retained its resistance, *i.e.*, its infectivity titre was not influenced by the presence of horse serum.

The IS line, on the other hand, retained its sensitivity, but did not lose its IR component, thus IR infectious units approximately unchanged in number could be selected from its population by passage in the presence of horse serum.

Table III

*Inhibitor sensitivity of IS and IR lines
of the Hong Kong/1/68 strain at different passage levels*

Passage No.	HI titre of heat-treated horse serum to line		Log infectious units per 0.1 ml allantoic fluid			
			IS line		IR line	
	IS	IR	IS	IR	IS	IR
1	1 : 16 000	10	7.8	2.0	?	6.7
3	1 : 8 000	10	8.0	2.3	?	6.9
5	1 : 16 000	10	7.6	1.8	?	6.6
10	1 : 12 000	10	7.8	2.1	?	7.1

?: could not be determined owing to the preponderance of IR virions

For further experiments, an artificial mixture of the IR and IS lines was subjected to serial passages in embryonated eggs and in mice. In the original mixture, the ratio of IS to IR virus was approximately 100 : 1. By the 14th egg passage, like in the experiment of CHOPPIN and TAMM, the predominance of the IS virus became more prominent, whereas in the mouse passage an inverse phenomenon occurred (Table IV).

Table IV

*The IS/IR ratio in the course of passages
started with a 100:1 mixture of IS and IR
infectious units*

Passage No.	IS/IR ratio after passages in	
	mice	eggs
1	100/1	100/1
2	100/1	125/1
5	10/1	2 000/1
14	1/1	10 000/1

Taking into consideration that the inhibitors in the serum of different animal species are different in both quality and quantity [11, 12], selection experiments were performed with heated fowl, mouse, guinea-pig and human sera. The practical aim of these experiments was to obtain an IR line suitable for use in the HI test. The titre of the IR component selectable by different sera from a preparation of the Hong Kong/1/68 strain appeared to be independent of the donor's species. Resistance of the IR virions obtained from different eggs at the limit dilution was, however, variable, and this variability was again independent of the serum used for selection. The IR lines which had been obtained from different eggs inoculated with the limit dilution were different in sensitivity. If such lines were subjected to serial egg passages without serum, they retained their initial resistance. It has therefore been concluded that the IR virion population of influenza A2 virus strains consists of virions of variable inhibitor-resistance.

It is known from literary data [7, 13] that even specific antibodies show a greater affinity to IS than to IR virions. However, the difference in this respect is less pronounced than in the case of the non-specific inhibitors. The question arises how IS and IR particles behave in the presence of both nonspecific inhibitor and antibody.

In the HI test the titre is dependent on the amount of the added virus. To elucidate the quantitative relations as regards both nonspecific and specific antibody, the HI activity of human nonimmune and convalescent sera was titrated in the presence of growing amounts of IS or IR virus.

According to Table V, the titre of the nonspecific inhibitor was much more influenced by the amount of the virus than by the amount of the antibody. The inhibitor and antibody titres of normal and immune guinea-pig serum as a function of the amount of virus are more clearly visualized in Fig. 1.

Erythrocyte receptors for IR and IS virus. The difference between the IS and IR variants manifests itself in relation of the erythrocyte receptor as well [7, 8]. This may be explained by the close chemical relation between the serum inhibitor and the erythrocyte receptor. To investigate this relation, determination of the dynamics of the adsorption to, and elution from, erythrocytes seemed to be an adequate method.

Thus, adsorption and elution of IS and IR lines were examined, using erythrocytes of various animal species, and IS and IR lines selected from the Hong Kong/1/68 strains. Two hundred units of virus mixed with an equal volume of a 0.5% erythrocyte suspension was kept at +4°C for 15 minutes. It was then centrifuged, and a negligible volume of supernatant was removed for HA titration. Subsequently, the erythrocytes were resuspended and then placed in a water bath of 37°C temperature for 60 minutes while the HA titre was determined in the supernatant of centrifuged samples taken at intervals. When the elution period had ended the erythrocytes were again

Table V

*Titration of negative and convalescent sera
with the IS and IR variants of the Hong Kong/1/68 strain*

Serum sample No.	Titres as tested with							
	4		8		16		32	
	haemagglutinating units of line							
	IS	IR	IS	IR	IS	IR	IS	IR
N* 1	120	—	40	—	10	—	—	—
N 2	480	10	80	—	20	—	—	—
N 3	320	—	60	—	20	—	—	—
N 4	640	20	240	10	60	—	20	—
Average	390		105		28		5	
Relative titre ⁺	100		27		7		1.3	
C* 1	3200	1200	2400	800	1000	600	800	480
C 2	2400	800	1600	600	1200	480	800	240
C 3	6400	2400	3200	1600	2400	800	1200	640
C 4	1200	640	800	320	480	128	320	64
Average		1260		830		452		356
Relative titre ⁺		100		66		36		28

N* = sera without homologous antibody

C* = convalescent sera

+ = in per cent of the titre obtained in the presence of 4 HA units

resuspended and the suspension was placed in a refrigerator of $+4^{\circ}\text{C}$. A significant re-adsorption of virus indicated that the elution was not accompanied by enzymatic destruction of the virus receptors, *i.e.*, the elution was mainly physical in nature.

The PR8 strain, a strain having no IR and IS variants, was also included in this series of experiments. Human, guinea-pig, horse and sheep erythrocytes were used. The adsorption and elution curves are shown in Fig. 2a, b, c.

The JS and IR lines of the A2 Hong Kong/1/68 strain agglutinated, and were adsorbed to, the erythrocytes of all the four species. However, elution of the IS virions at 37°C was relatively slow, from human and guinea-pig erythrocytes it was only partial. From the latter hardly any IS virus was recovered. At 4°C , re-adsorption occurred in each combination. However, the amounts of IR virus re-adsorbed to human and guinea-pig erythrocytes during the 15-minute period were almost negligible and re-adsorption of the

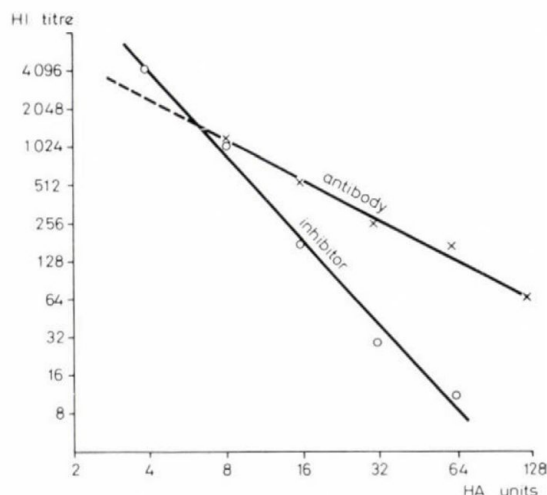


Fig. 1. Inhibitor and antibody titre of guinea-pig immune serum as a function of HA units of virus in the HI test

same line to horse and sheep erythrocytes was also partial. Re-adsorption of the IS line approximated the degree of the first adsorption.

According to the results of these experiments, total elution of the IR virions and the total or partial elution of the IS virions should be attributed to a direct temperature effect rather than to enzymatic destruction (Fig. 2b, c).

The antigenic identity of the IS and IR lines. IS and IR variants of seven influenza A2 strains isolated in Hungary in the period 1957 to 1964 were included in this experiment. Immune serum was produced to each line in roosters and cross HI tests were carried out separately between the IR lines and between the IS lines. The difference between strains is expressed in d values [14]. (The greater the d value the greater the antigenic difference.)

In the two triangles of Fig. 3 the corresponding d values are in good agreement, while most of the d values in the IR triangle are slightly higher than the corresponding values in the IS triangle.

In further experiments, anti-IS sera were re-adsorbed with IR virions of the same strain and *vice versa*. No residual antibody could be detected with the homologous line in the adsorbed sera. Since, according to earlier experiments [15, 16] very slight antigenic differences can be detected by the same cross absorption method, there is no doubt that the IS and IR lines of the same influenza A2 strain are identical antigenically.

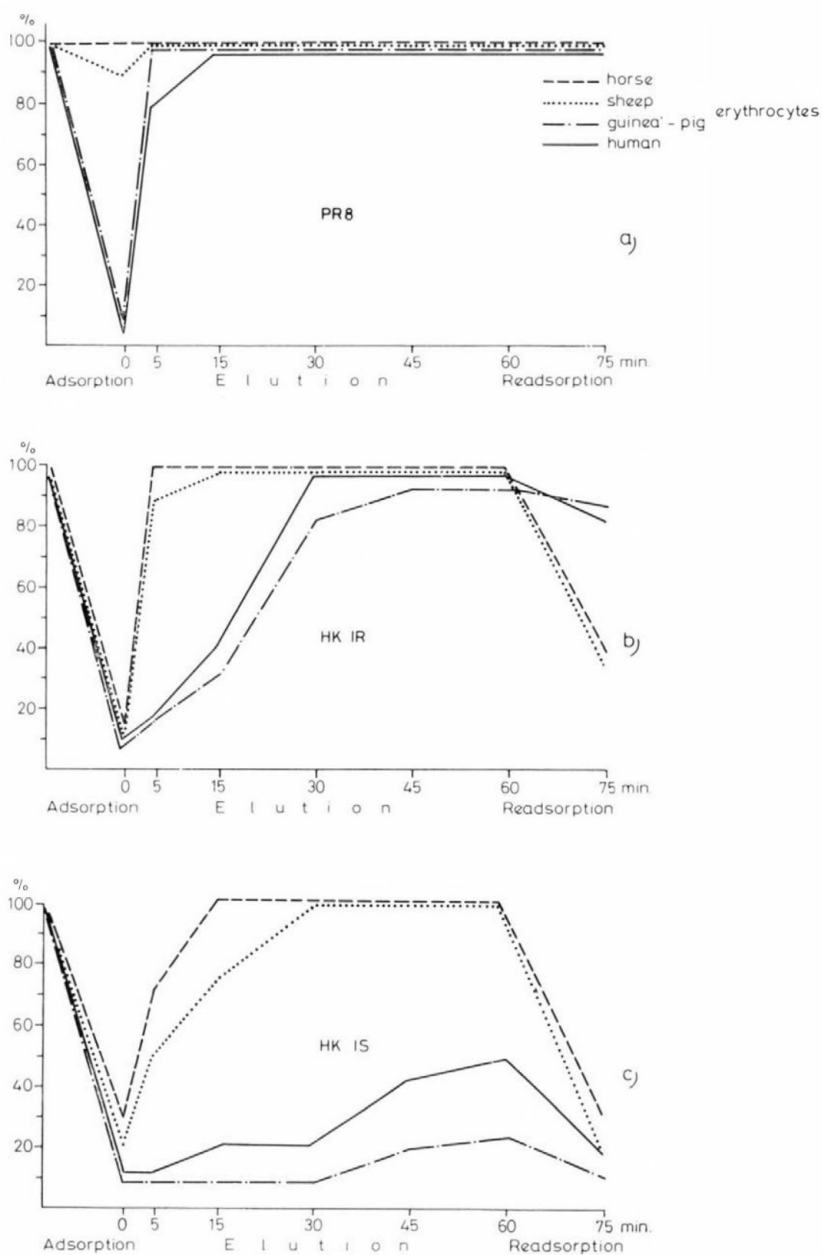


Fig. 2. Adsorption—elution—readsorption curves

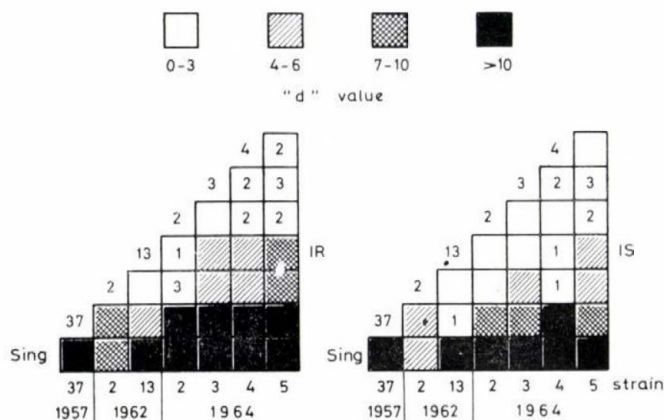


Fig. 3. Strain-differences expressed in d values

Discussion

Based on the results of selection experiments, we suppose that the IS virion carries the basic characteristics of the influenza A2 subtype and that the IR virion is its mutant. IR virions arise when apparently pure IS lines are serially passaged at limit dilutions in embryonated eggs. The rate of their formation appeared to be almost invariable in different passages of the same strain, but showed strain differences in the range of the mutation rates generally found in other systems. In the lack of an available method, on the other hand, we cannot answer the question whether IS virions arise in the course of passages of IR lines. It may be considered an indirect evidence of the lack of such a mutation that the IR lines retained their IR property through several passages in embryonated eggs, though the IS mutant became predominant after several passages started with artificial mixtures of IR and IS virions.

The serial passage in mice of an artificial mixture of IR and IS virions supplies some information why the IR character predominated in the population of numerous strains isolated in 1959. Supposedly, the nonimmune host favours the multiplication of the IR mutant and, in the early period of the A2 era, specific immunity to A2 virus was poor in the human population. Since, however, herd immunity had developed, the multiplication of the relatively less pathogenic [17] IR virus has been hindered to a greater degree and, consequently, all the subsequent isolates have been of IS character.

Our experimental results are consistent with the view that the IS virion is avian, in addition to the mucoprotein inhibitors of the earlier influenza virus strains, for an inhibitor of protein nature (γ inhibitor). Since the γ inhibitor is not destroyed by the neuraminidase enzyme of the virus, the stable

blocking of the haemagglutinin results in neutralization of the infectivity. It is due to the stability of this binding that the γ inhibitor is readily adsorbable from the serum [18] even more rapidly than the simultaneously present antibody is absorbed. Therefore, cross serum absorption tests are not disturbed by the inhibitors.

It should be stressed that mucoprotein receptor of the erythrocytes is attacked by the neuraminidase of both the IS and IR virions [7, 19]. The inhibitor-resistance or sensitivity of a strain is due to the haemagglutinin, owing most probably to its steric configuration. This configuration is responsible for the binding of the γ inhibitor and for species differences in this respect.

Supposedly, the steric configuration of the inhibitor is modified by a polysaccharide component [20], which, however, in contrast with the mucoprotein inhibitor, is not destroyed by the virus enzyme.

The assumed steric difference in the IR and IS haemagglutinins causes no antigenic difference, suggesting that the tertiary configuration of the haemagglutinin or immunologically indifferent side chains play the main role in the virus-inhibitor binding.

A similar explanation may be offered for the interaction between IS and IR virions and erythrocyte receptors. Both virions are bound by the mucoprotein receptor in the same manner, and this binding ceases when the mucoprotein receptor has been destroyed by the viral neuraminidase. However, elution of the IS virions is slowed down or even prevented by protein receptors capable of a stable reaction with these virions. Since the protein receptors, like the serum inhibitors, vary from species to species, elution of the IS variant is dependent on the species of the erythrocyte donor (Fig. 2c).

The variability of the haemagglutinin manifests itself in some differences in further biological characteristics of the IS and IR lines. These are summarized in Table VI on the basis of our investigations and literary data.

In addition, BEARE [17] found that in humans the IR virion was less reactive than the IS virus. We found no significant difference in adaptability to mouse and in mouse pathogenicity between the two lines and also in the production of the von Magnus-type incomplete virion.

Table VI
Characterization of IS and IR lines

Characteristics	IS line	IR line
Mean HI titre of normal sera		
human, untreated	250	
heat-treated	5 000	
guinea-pig, untreated	2 000	
heat-treated	20 000	10
horse, untreated	1 000	
heat-treated	20 000	
mouse, untreated	0	
heat-treated	10	
Neutralization with inhibitor	successful	unsuccessful
Neuraminidase activity	+	+
Elution from erythrocytes	no or partial	partial or full
HA with KIO ₄ or RDE-treated erythrocytes	+	—
IS/IR ID ₅₀ in the original strains	10 ⁸ /10 ⁷	10 ⁷ /10 ⁷
IS/IR ID ₅₀ in the selected lines	10 ⁸ /10 ¹ —10 ³	?/10 ⁷
Mean HA titre in egg	567	260
Mean egg ID ₅₀ log	7.7	6.8

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AUSTRALIA ANTIGEN SURVEY IN HUNGARY

II. SEX AND AGE DISTRIBUTION OF ANTIGEN-POSITIVE CASES OF ACUTE HEPATITIS AND CLINICAL AND LABORATORY STUDIES

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Summary. The distribution by age and sex of 690 Australia-antigen positive patients, aged 16 years or more, suffering from acute viral hepatitis showed a preponderance of males (61%), and of young adults of both sexes. As compared to the age distribution of the general population the relative frequency of the positive patients over 50 years of age was higher than expected. These data are discussed in comparison with the age and sex distribution of blood donors reported in the first paper of this series.

The clinical-laboratory and serological results obtained for 300 adult patients on the first day after admission were analysed comparatively. All patients admitted with an SGPT value of 600 Wroblewski units or higher in a given period of 1971 were included in the study. Australia antigenaemia was demonstrated by the complement-fixation test in 91% and 70% of the cases diagnosed clinically as serum and infectious hepatitis, respectively. Seventy-one per cent of the patients with antigenaemia and 35% of those without antigenaemia had anticomplementary sera. Only 16% of the former had neither free antibody nor anticomplementary activity in their serum. Antigenaemia showed no correlation to SGPT activity and serum bilirubin level.

On the basis of 66 000 immunoelectroosmophoresis (IEOP) tests we have demonstrated that 1.0% of the blood donors in Hungary had Australia (Au) antigenaemia in 1971 [1]. Antigenaemia was more frequent in male than in female donors and it was more frequent in young than in elder male donors.

In the present work the distribution by sex and age of 690 adult patients with Au-positive acute hepatitis was established and correlation was sought between the degree of Au antigenaemia on the one hand and the SGPT activity and the serum bilirubin value on the other.

Materials and methods

Cases. Sixhundred and ninety patients admitted with acute hepatitis in the years 1970 and 1971 were analysed for age and sex distribution. Only those cases were included in this group whose serum samples contained Au antigen detectable by the complement fixation (CF) test. In this analysis no clinical distinction has been made between serum hepatitis (SH) and infectious hepatitis (IH).

For a comparison of the clinical-laboratory and serological results 300 patients were selected who had been admitted with an SGPT value of over 600 Wroblewski units in the period January to December, 1971. The patients who had received transfusion or serial injections

in the 2 to 6-month period preceding admission were regarded as suffering from SH. The clinical diagnosis was IH in all the remaining cases. In this group the patients whose early serum sample contained no Au antigen detectable by the CF test were considered negative.

Laboratory methods. The methods used have been described in the first paper of this series [1].

Results

Distribution by sex and age of 690 Au-antigen-positive patients. Distribution by sex: males, 423 (61%), females, 267 (39%). Distribution by age is shown in Fig. 1 in five-year age groups. The number of 16 to 19-year-old male patients was high (46), especially if it is taken into consideration that this group included only four cohorts. Strikingly few male patients belonged to the 20 to 24-year age group, supposedly because a considerable proportion of these men were doing their active military service and we did not receive materials from military hospitals. Data for the 25 to 49-year age group fluctuated around the average value, but there was a considerable rise over 50 years. In the higher age groups the curve declined at a slower rate than expected on the basis of the age distribution of males in the general population. The decline was well-defined only from 70 years upwards.

The age distribution of the female patients showed a less pronounced fluctuation, although the smaller number of female patients in the present study would have allowed wider fluctuation. Apart from this, the tendencies were similar to those seen in the group of male patients.

SGPT and serum bilirubin levels of SH and IH patients. On the basis of their history, 137 (46%) of the thoroughly investigated 300 patients were regarded as suffering from SH, in the other cases (163, i.e., 54%) the clinical

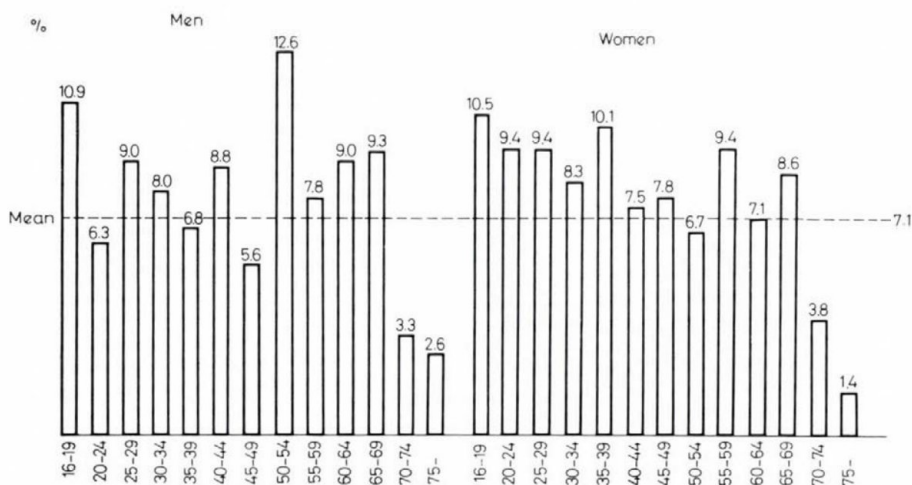


Fig. 1. Age distribution of Au-antigen-positive patients

diagnosis was IH. Both groups were subdivided into three subgroups according to the SGPT level in their early serum sample (Table I). The distribution of these subgroups seemed to be independent of the clinical diagnosis (IH or SH). On the other hand, high (10 mg/100 ml) serum bilirubin values were somewhat more frequent in the SH than in the IH group (47% and 21%, respectively). As expected, some positive correlation between SGPT and serum bilirubin values was observable in both groups.

Au antigenaemia of SH and IH patients. Of the 163 IH patients, 114 (70%), of the 137 SH patients, 124 (91%) had antigenaemia at the time of sampling. As compared to literary data, both percentages were high. This may have been due to the high sensitivity of the CF test and to the fact that only patients with high SGPT values (≥ 600 Wroblewski units) were included in the study. In spite of these, antigenaemia was detectable in 70% of patients without any previous parenteral intervention.

SGPT activity and serum bilirubin vs. Au antigenaemia. Considering that in the present material SGPT activity showed no relation to the clinical diagnosis, we did not separate the cases of IH and SH in further analysis.

Table I

SGPT activity and serum bilirubin level in acute, clinically SH and IH cases

Clinical diagnosis	SGPT*	Serum bilirubin mg/100 ml			Total	
		<5.0	5.0-9.9	≥ 10.0	No.	per cent
IH	600-999	11	33	2	46	28
	1000-1399	8	30	11	49	30
	≥ 1400	7	40	21	68	42
Total		26 (16%)	103 (63%)	34 (21%)	163	100
SH	600-999	8	18	12	38	28
	1000-1399	4	20	9	33	24
	≥ 1400	2	21	43	66	48
Total		14 (10%)	59 (43%)	64 (47%)	137	100

* Wroblewski units

In Table II the cases are divided into three groups by the degree of antigenaemia (negative; weakly and strongly positive) and also by SGPT value. No correlation was demonstrable.

As Table III shows, there was no correlation between the serum bilirubin level and the degree of antigenaemia.

Table II

SGPT activity and Au antigenaemia in cases of acute hepatitis

SGPT*	Cases		Au-negative cases		Au-positive cases with CF titre		Total Au-positive		G. M. of titres
	No.	per cent	No.	per cent	1 : 5 - 1 : 20	≥ 1 : 40	No.	per cent	
600—999	84	28	18	29	33	33	66	28	25
1000—1399	82	27	19	31	31	32	63	26	27
≥ 1400	134	45	25	40	57	52	109	46	24
Total	300	100	62	100	121	117	238	100	25

* Wroblewski units

Table III

Serum bilirubin and Au antigenaemia in cases of acute hepatitis

Serum bilirubin mg/100 ml	Cases		Au-negative cases		Au-positive cases with CF titre		Total positive		G. M. of titres
	No.	per cent	No.	per cent	1 : 5 - 1 : 20	≥ 1 : 40	No.	per cent	
< 5.0	40	13	7	11	16	17	33	14	22
5—9.9	162	54	41	66	66	55	121	51	24
≥ 10.0	98	33	14	23	39	45	84	35	27
Total	300	100	62	100	121	117	238	100	25

Anticomplementary activity and antibody content. Among the 238 patients with antigenaemia 33 (13%) had free anti-Au antibodies. The antibody titres did not exceed the 1 : 8 level and it occurred rarely that the difference between antibody titre and anticomplementary activity was more than one dilution step. A hundred and ninety sera (63%) showed anticomplementary activity. Of these, 22 were antigen-negative, i.e., 35% of the antigen-negative sera were anticomplementary. On the other hand, 71% (168) of the antigen-positive sera proved to be anticomplementary. Only those anticomplementary sera were accepted as positive whose antigen titre exceeded its anticomplementarity by at least two dilution steps. Neither free antibody nor anticomplementary activity could be demonstrated in 38 of the 238 antigen-positive sera (16%). This was in sharp contrast with the lack of antibody and anticomplementarity in the sera of antigen-positive donors [1].

Discussion

It seems of interest to compare the age and sex distribution of the present 690 patients with that of the donors found by us Au-antigen-positive in approximately the same period of time [1]. There appears some parallelism between the two distribution curves.

(i) The frequency of Au antigenaemia was higher in the male donors than in the female ones and in the present material males represented the majority (61%) of the antigen-positive patients;

(ii) the relative participation of the youngest age group (16 to 19 years and 18 to 19 years, respectively) was the highest in both materials;

(iii) the preponderance of the youngest age groups was more pronounced in males than in females in both materials.

The high participation of the age groups over 50 years in the Au-positive patients as compared to the age distribution of the general population is consistent with the fact that in Hungary in recent years the age-specific hepatitis morbidity for these age groups has been considerably higher than that for the age groups from 30 to 49 years. The high frequency of Au-antigen-positive hepatitis in the 16 to 19-year age group may also be related to the higher age-specific morbidity, but it should be taken into consideration that the excess morbidity in these age groups is usually attributed to IH. These findings support the view, much debated in literature [2-10], that part of the non-parenteral IH cases is caused by a virus identical with, or closely related to, the hepatitis B virus. The same view is supported by the fact that 70% of the clinically IH cases proved to be Au-antigen-positive.

BLUMBERG [11] accepts the viral nature of the agent carrying the Au antigen, but at the same time assumes that the antigen itself is a genetically determined lipoprotein polymorphism. Accepting this hypothesis, it must be assumed that those who possess the gene (even in a repressed state) governing the production of the isoantigen should recognize the infectious agent as their own and give no immune response to it. Such subjects may become chronic virus carriers, or eventually develop chronic illness. Sixteen per cent of our Au-antigen-positive patients had developed neither anti-Au antibodies nor anti-complementary activity by the time of blood sampling, which had been preceded by a long incubation period on several days of manifest illness. These or at least part of these, as well as the great majority of the Au-positive donors examined by us, might be considered as belonging to this group.

The age-specific frequency of Au-antigenaemia in donors has been discussed in a previous report [1] in view of the genetical concept and other (hormonal, environmental, *etc.*) factors. The approximate parallelism demonstrated in the present work seems to be in accordance with those points.

In the present material there was no correlation between the SGPT activity and the presence and serum titre of the Au antigen in the early phase of hepatitis. It should, however, be taken into consideration that the patients with SGPT values less than 600 Wroblewski units were excluded from this study. Thus, we may only conclude that even higher SGPT values are not related to the aetiological role of the hepatitis B virus.

There was no appreciable correlation between Au antigenaemia and SGPT value. As expected, some correlation was demonstrated between SGPT and serum bilirubin values.

It should be noted that the lack of a correlation between the parameters under study is only valid for the early stage of hepatitis. Follow-up studies [12] may result in well-defined correlations.

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SUBDIVISION AND CORRELATION STUDIES OF SEROLOGICALLY GROUPED *ESCHERICHIA COLI* STRAINS BY PHAGE TYPING, COLICINOGENY, LYSOGENY AND BIOCHEMICAL TEST

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Summary. For phage typing of *Escherichia coli* strains, a phage set of 28 phages was used to characterize the phage patterns and phage groups. The serogroups and serotypes were compared with the phage patterns and phage groups in laboratory strains of *E. coli* and in strains isolated from patients suffering from enteric and extra-enteric diseases. Besides studying phage sensitivity, colicinogenic and lysogenic properties were determined and the β -phenyl propionic acid reaction of D'ALESSANDRO and COMES was performed. This combined method allowed to establish the pathogenic role of *E. coli* strains not identified or unidentifiable by serological tests. The subsequent serological identification of strains considered identical on the basis of their phage patterns, colicinogeny, lysogeny and PP reaction was easier to perform than to carry out random serotyping.

The results showed that the serotype might be deduced from the phage pattern, for instance, the phage pattern characteristic of serogroup O4 was 4, 17, 20, 22; and of serogroup O18, the pattern was 4, 12, 13, 16, 17, 18.

Phage pattern, colicinogeny, lysogeny and the PP reaction could be used for a further differentiation among the strains of serogroups O18 and O111. In serogroups O4 and O8, phage pattern and colicinogeny while in serogroup O124, lysogeny and colicinogeny allowed a further subdivision. The reliability of typing was controlled on material collected from various hospitals. This showed that the strains probably originating from a common source were identical on the basis of their phage pattern and phage groups. Colicinogeny and lysogeny proved to be properties less stable than phage sensitivity.

Data on the pathogenicity of *Escherichia coli* mostly refer to, and review the serotypes of, *E. coli* strains causing enteritis in infants, children, and adults. The observations of EWING *et al.* [1], EDWARDS and EWING [2], COSTIN [3], LACHOWICZ [4], SAKARAKI *et al.* [5], NOVGORODSKAYA [6], and ØRSKOV *et al.* [7] show that the enteropathogenic strains of *E. coli* can be divided into 44 O serogroups and 234 K and H serotypes. First the pathogenic role of *E. coli* strains causing infectious gastroenteritis in infants was established and consequently a finer intraserotypic subdivision of these strains has become necessary for the elucidation of epidemiological conditions.

Of the *E. coli* strains originating from extraenteral diseases, a subdivision of those from urinary tract infections was attempted according to the phage types. UJVÁRY [8], RANTZ [9] and KUNIN [10] found that some 17 kinds of serotypes were responsible for these infections and that the organisms were usually different from those causing enteritis in infants.

The pathogenic role of *E. coli* strains has been established by serological methods, but a number of diagnostic and epidemiologic problems are still unclear as to *E. coli* strains causing enteric or extra enteric diseases. Clarification of these problems appears to require more than the usual biochemical and serological methods, as the former offer little possibility for a type subdivision while serological methods can reliably be performed but in a few laboratories. Consequently, elaboration of a method for the phage typing of *E. coli* strains originating from pathogenic processes similar to that used for certain other groups of *Enterobacteriaceae*, viz. *Salmonella*, *Shigella*, *Klebsiella*, appeared to be interesting.

In a previous work [11–13], type-phages used for the determination of the phage types of strains O111, O55 and O26 were isolated from the faeces of patients suffering from enteritis. In the course of this study, *E. coli* strains different from those identifiable by routine tests could be isolated with a high frequency. At the same time, phages affecting these *E. coli* strains were also isolated. Of the several hundred phages isolated, 28 were selected on the basis of the phage sensitivity of *E. coli* strains. These 28 phages were used in an attempt to set up a phage series suitable for the identification of *E. coli* strains originating from the same source.

In order to develop the system of phage sensitivity, the phages have been classified serologically and their lytic activity has been studied.

Besides the phage sensitivity of the serologically identified strains, their colicinogenic and lysogenic properties, their capacity to induce haemolysis, and their behaviour in the β -phenyl propionic acid reaction (PP reaction) of D'ALESSANDRO and COMES [14] has been studied.

Materials and methods

Selection and classification of type-phages. Type-phages were isolated from the faeces of patients (mostly infants) suffering from enteric infections. The methods of isolation and propagation were the same as described previously [15]. *E. coli* strains of known antigenic structure and pathogenic according to data in the literature, were used as indicator strains for the selection of type-phages. The type-phages were classified according to their serological properties and lytic activity.

The phages propagated and the strains used for determination of the lysis spectrum and for propagation of the phages are shown in Table I. In addition to *E. coli* phages, two *Salmonella* phages were also used: Vi phage I+IV and phage PO containing phages O1, O2 and O3. Lysis with *Salmonella* phages is indicated in the tables by "S".

Strains. The 26 *E. coli* strains used for propagation and lysis spectrum as well as those isolated in different laboratories (a total of 162 strains) were serogrouped and serotyped by Drs IDA and FRITS ØRSKOV at the International Escherichia Centre of WHO. The O, K and H type strains maintained in the Culture Collection of the National Institute of Public Health (a total of 231 strains) were also studied while 16 O111 : 58B : - strains were supplied by Dr. H. RISCHE (Wernigerode, GDR). Thus, the serotype and the phage pattern were compared in a total of 435 *E. coli* strains.

Serological studies. Anti-phage sera were produced against each of the 28 type-phages. The neutralizing effect of the anti-phage sera was determined for every phage, and, in the case of neutralization, the K value of the sera was estimated according to BURNET *et al.* [16].

Table I

Type-phages, propagating and lysis-spectrum strains

Phages	Propagating strains	Antigenic structure
F 1	C 1	85 : + : —
F 2	C 29	19, 133 : + : 16
F 3	C 31	Rough : + : ?
F 4	O8	8 : + : +
F 5	O5	9 : + A : 9
F 6	C 23	25 : + : ?
F 7	C 32	79 : + : 2
F 8	F 42	26 : 60B : +
F 9	C 26	20 : + : —
F 10	111/1	111 : 58B : —
F 11	111/5	111 : 58B : —
F 12	C 35	125 : + : 25
F 13	C 8	18ac : + : —
F 14	C 15	77 : + : .
F 15	C 20	35 : + : 9
F 16	C 19	18ac : + : —
F 17	O58	18ac : 77B : —
F 18	O58	18ac : 77B : —
F 19	55/1	55 : 59B : 6
F 20	55/4	55 : 59B : 6
F 21	111/2	111 : 58B : 2
F 22	C 3	4 : + : 5
F 23	C 7	15 : + : —
F 24	O18	18ab : + : 14
F 25	C 9	18ab : + : 14
F 26	C 17	1 : 1 : 7
F 27	F 42	26 : 60B : +
F 28	C 24	Rough : + : 2

Determination of the phage types. The *E. coli* strains to be typed were inoculated from 24-hour agar plates into broth and after 2 and a half hours incubation, the cultures were poured over agar plates the excess being drawn off. After the cultures had dried, type-phages were dropped on the plates by a pipette used specially for this purpose or by means of a phage-typing apparatus (Biddalphy and Co., England). When dried, the plates were incubated at 37°C for 6 hours and then read. Lysis was registered according to intensity. The results were given in terms of lysis pattern referring to phages causing lysis. The phages were used at RTD (routine test dilution) estimated by titration in advance. RTD was defined as the highest dilution of phages causing a half-confluent lysis with the propagating strains. The phage pattern of the strains was characterized by listing the phages causing lysis (denoted with Arabic numerals). Phages giving lysis weaker than two crosses (less than 50—100 plaques)

are indicated in brackets. Besides phage patterns, phage groups (denoted by capital letters) are given, indicating the serogroups of the phages giving lysis (Fig. 1).

Media. Broth and solid agar media.

β -Phenyl propionic acid reaction. The reaction was carried out according to D'ALESSANDRO and COMES [14].

Colicinogeny. Colicinogeny studies were performed according to a modified method of ABBOTT and SHANNON [17]. *E. coli* strains plated on agar plates were inoculated into broth and incubated at 37°C for 24 hours. One drop of each strain to be studied was dropped in plates in triplicate. On every plate, 7 strains were studied. After 16 hours incubation at 37°C, the plates were exposed to chloroform vapour for 30 minutes and then the chloroform was evaporated. Finally, two-hour broth cultures of 3 indicator strains were mixed with soft agar (0.5 ml with 4 ml soft agar) and then layered over plates. The inhibition zones were read after an incubation at 37°C overnight. The type of colicin was characterized by listing the indicator strains surrounded by inhibition zones. As indicator strains, *E. coli* K12, *Sh. sonnei* 17 (Abbott-Shannon), and *E. coli* Phi (Mac Geachie) were used (Fig. 2).

Lysogeny. The 3 indicator strains mentioned above were used for studying lysogeny. The test strains were killed by chloroform. The phage isolated from the lysogenic strains was characterized by listing the indicator strains in which it caused lysis.

Results

Classification of the 28 type-phages according to their serological properties is shown in Table II. The type-phages could be divided into 7 serogroups including 14 subgroups. The serological groups were set up according to the lysis-neutralization properties of the antiphage sera produced with the type-phages. Table II shows the highest dilution of the sera giving complete lysis neutralization.

The lysis spectra of the test strains were determined with the phages classified according to their RTD. The lysis patterns obtained are shown in Table III.

The results were evaluated according to the O antigens and serogroups of the strains. Phage pattern, phage groups, colicinogeny, lysogeny, PP reaction and haemolysis for each serotype are presented in the Tables.

Special Tables are devoted to the results for the strains classified according to the O antigen, which have been isolated with a higher frequency and which probably played a role in pathogenicity, and yielded results allowing the formulation of some general rule. The results obtained for *E. coli* strains originating mostly from the culture collection are shown in the Tables on the basis of the O antigen. Of the strains belonging to the culture collection, only a small number was studied in each serogroup and thus for the time being no general conclusion concerning serogroups has been drawn from these results.

In the Tables the column "Source" shows the different hospitals where the material yielding the strain had been obtained from, and the clinical diagnosis.

The results found for strains belonging to serogroup O4 are shown in Table IV. Part of the strains collected in the same hospital exhibited identical

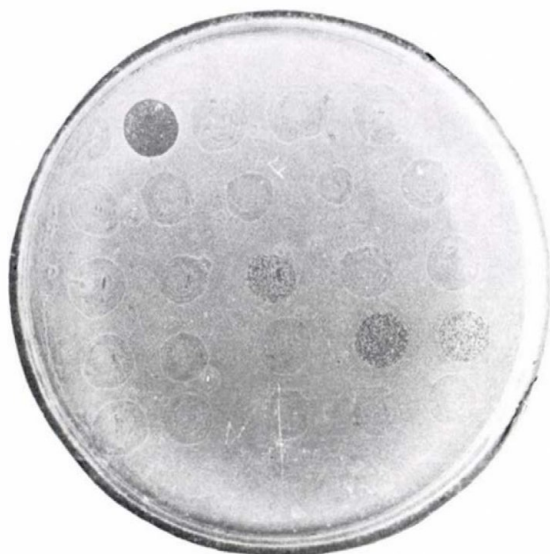


Fig. 1. Phage pattern of strain 18ac : 77 : 7 (3219/54). Phage pattern: 4, 12, 16, 17; phage group: A1, A4, B2

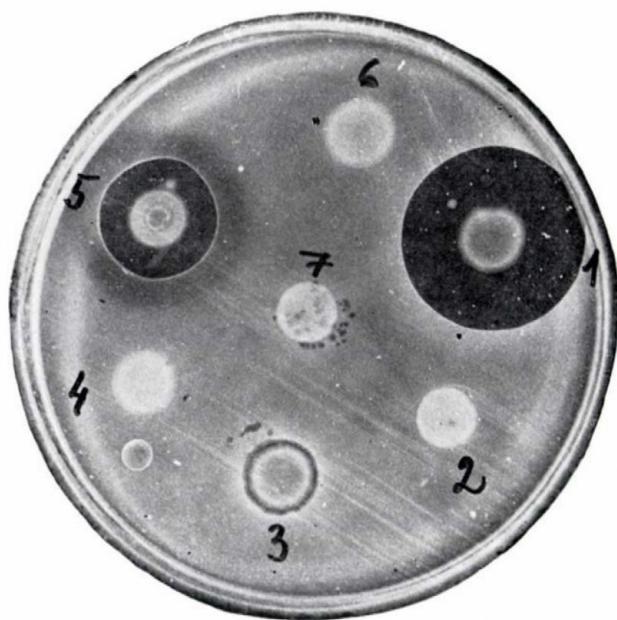


Fig. 2. Colicinogenic and lysogenic property of 7 *E. coli* strains on *E. coli* K12 as indicator strain. Colicinogenic ++ and +: strains Nos 1 and 5. Colicinogenic +: strain No. 3 (in brackets). Lysogenic: strains Nos 3 and 7. Negative: strains Nos 4 and 6

Table II
Neutralization titre of antiphage sera

Sero-group	Type phage	A ₁				A ₂			A ₃				A ₄		B ₁		B ₂				B ₃	B ₄	C ₁	D ₁	E ₁	F ₁	F ₂	G ₁	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
A ₁	1	10 ²	10 ¹	10 ²	10 ³	10 ²	10 ²	10 ²	10 ²	10 ¹	10 ¹	10 ¹																	
	2	10 ¹	10 ³	10 ⁴	10 ²	10 ²	10 ¹	10 ²																					
	3	10 ¹	10 ³	10 ⁶	10 ⁴	10 ²	10 ²	10 ²	10 ¹																				
	4	10 ¹		10 ²	10 ⁴																								
A ₂	5	10 ¹	10 ²	10 ²	10 ³	10 ³	10 ¹	10 ³																					
	6	10 ¹	10 ¹	10 ⁴	10 ¹	10 ²	10 ²	10 ²	10 ¹																				
	7	10 ¹	10 ³	10 ⁴	10 ³	10 ²	10 ²	10 ³																					
A ₃	8	10 ¹	10 ¹	10 ²	10 ⁴				10 ³	10 ²	10 ²	10 ³																	
	9	10 ¹	10	10 ¹	10 ²				10 ³	10 ²	10 ²	10 ²																	
	10	10 ¹	10	10 ²	10 ³				10 ³	10 ²	10 ²	10 ³																	
	11	10 ¹		10	10 ²				10 ²	10 ¹	10 ²	10 ³																	
A ₄	12		10	10 ³	10 ¹	10 ¹							10 ³	10															
	13												10 ²																
B ₁	14													10 ²															
	15				10 ¹										<10	10 ³													
B ₂	16																10 ⁴	10 ²	10 ²										
	17																10 ³	10 ²	10 ²										
	18																		10 ³	10 ²									
	19								10									10 ¹	10 ¹	10 ²									
B ₃	20																		<10	10 ⁴									
B ₄	21																				10 ⁴								
C ₁	22																					10 ²							
D ₁	23																						10 ³	10 ¹					
	24																						10 ²	10 ³					
E ₁	25																								10 ⁴				
F ₁	26																									10 ⁴	10 ¹		
F ₂	27																										10 ²		
G ₁	28																												10 ⁴

phage patterns. Strains isolated from the trachea of premature babies suffering from interstitial pneumonitis, from the urine of a patient having cystitis, and from the faeces of infants suffering from enteritis, showed 6 different phage patterns. Part of the strains was sensitive only to phage 4 while most of them were sensitive to phages 4, 17, 20 and 22. The phage group characteristic of serogroup O4 was A₁, B₂, B₃, C₁.

The strain with the antigenic structure of 4 : 52L :— displayed a phage pattern different from that of the other strains and it was sensitive besides *E. coli* type phages also to *Salmonella* phage "PO" (phages O₁, O₂, O₃ mixed = PO). Thus antigen O4 of *E. coli* may contain a component related to the O antigen of *Salmonella* the occurrence of which has been described for a number of other *E. coli* strains. Of the strains studied, 3 were colicinogenic, none was lysogenic, 12 induced haemolysis, and all were PP negative. On the basis

Table III

Lysis spectrum of E. coli type phages
(Type phages at RTD)

		Serogroup of type - phage																												
		A ₁		A ₂		A ₃		A ₄		B ₁		B ₂		B ₃	B ₄	C ₁	D ₁	E ₁	F ₁	F ₂	G ₁									
Test strain	Antigenic structure	Type phage																												
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
C ₁	85 : - -	Cl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
C ₂₉	19, 133 : - 16	-	Cl	-	-	-	-	-	scl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
C ₃₁	Rough : - ?	-	scl	Cl	-	-	-	Cl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
O ₈	8 : - 1 :	-	-	-	-	Cl	Cl	-	scl	-	-	-	-	Cl	Cl	-	±	scl	-	-	-	-	-	-	±	-	-	-	-	
C ₅	9 : (A) : 9	-	-	-	-	-	Cl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
C ₂₃	25 : - ?	-	scl	scl	-	-	Cl	scl	-	-	-	-	-	-	-	-	-	scl	-	-	-	-	-	-	-	-	-	-	-	
C ₃₂	79 : - 2	++	Cl	scl	-	-	±	Cl	scl	scl	+++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
F ₄₂	26 : 60 (B) : -	scl	-	+	-	-	scl	-	Cl	scl	++	-	scl	-	-	Cl	-	-	-	-	-	-	-	-	-	-	-	Cl	scl	
C ₂₆	20 : - -	++	-	±	-	-	-	-	scl	Cl	++++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
111/1	111 : 58 (B) : -	±	-	-	-	-	-	-	±	++	Cl	±	-	-	-	-	-	-	-	-	-	Cl	-	-	-	-	-	-	-	
111/5	111 : 58 (B) : -	-	-	-	-	-	-	-	Cl	+++	-	Cl	Cl	-	-	-	-	-	-	-	-	-	-	±	Cl	-	-	-	Cl	
C ₃₅	125 : - 25	-	-	-	-	-	-	scl	-	-	-	-	-	Cl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
C ₈	18ac : - -	-	-	-	scl	-	-	-	-	-	-	-	-	Cl	-	-	-	OL	scl	-	-	-	-	-	-	-	-	-	-	
C ₁₅	77 : - ?	-	-	-	-	-	-	-	+++	scl	++	-	scl	-	Cl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
C ₂₀	35 : - 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cl	-	-	-	-	-	-	-	-	-	-	-	-	-	
C ₁₉	18ac : - -	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cl	Cl	-	-	-	-	-	-	-	-	-	-	-	
O ₅₈	18ac : 77 (B) : -	-	-	-	++	-	-	-	-	-	-	-	scl	Cl	-	-	-	Cl	Cl	-	-	-	-	-	-	-	-	-	-	
55/1	55 : 59 (B) : 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cl	scl	-	-	-	-	-	-	-	-	
55/4	55 : 59 (B) : 6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cl	-	-	-	-	-	-	-	-	
111/2	111 : 58 (B) : 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	scl	-	-	-	-	-	-	-	
C ₃	4 : - 5	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+++	-	Cl	-	-	-	-	-	-	-	
C ₇	15 : - -	-	-	-	Cl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cl	-	-	-	-	-	-	
O ₁₈	18ab : - 14	-	-	-	-	-	-	-	-	-	-	-	scl	-	-	-	-	-	-	-	-	-	-	Cl	-	-	-	-	-	
C ₉	18ab : - 14	-	-	-	scl	-	-	-	-	-	-	-	scl	-	-	-	-	-	-	-	-	-	-	-	Cl	-	-	-	-	
C ₁₇	1 : 1 : 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Cl	-	-	-	
C ₂₄	Rough : - 2	-	scl	+	-	-	-	-	-	+++	+	-	-	-	-	scl	-	-	-	-	+++	-	-	-	-	-	-	-	-	Cl

of antigens K and H, the serotypes showed no close correlation with the phage pattern as the phage pattern of strains with the same K and H antigens was different.

The data obtained for the 31 strains belonging to serogroup O₈ are shown in Table V. The O, K, H antigens and the phage pattern of the strains of serogroup O₈ exhibited heterogeneous patterns. A total of 15 strains from different hospitals was studied. Most strains isolated from patients were obtained from respiratory or from respiratory and enteric infections. The strains isolated from the trachea and faeces collected in Hospital 2 were identical in phage pattern, colicinogeny and PP reaction. Of the strains studied from serogroup O₈, 7 were colicinogenic of which 5 belonged to phage resistant strains. Only two lysogenic strains were found. Neither induced haemolysis and the PP reaction was also negative in every case. The strains of the same

Table IV

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of E. coli serogroup O4

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
4 : 3L : 5	4	A1	—	—	+	—	1	Culture collection	W 4/41
4 : 3 : —	4	A1	—	—	+	—	2	Hosp. 1, faeces, enteritis	C87
4 : 3 : —	4, 17, 18, 20, 22	A1, B2, B3, C1	—	—	+	—	1	Hosp. 1, faeces, enteritis	C117
4 : 6L : 5	4, 17, 22	A1, B2, C1	17, K12	—	—	—	1	Culture collection	Bi 7457/41
4 : 12L : —	4, 20, 22	A1, B3, C1	—	—	—	—	1	Culture collection	Su 65/42
4 : 12 : 1	4, (20), 22	A1, B3, C1	Phi, K12	—	+	—	1	Hosp. 1, faeces, enteritis	C53
4 : 12 : 1	4, 17, 20, 22	A1, B2, B3, C1	—	—	+	—	2	Hosp. 1, faeces, enteritis (1); Hosp. 2, trachea, interstitial pneumonia (1)	C62
4 : 12 : 5	4, 17, 20, 22	A1, B2, B3, C1	Phi, K12	—	+	—	1	Hosp. 1, faeces, enteritis	C11
4 : · : —	20, 22	B3, C1	—	—	+	—	1	Hosp. 3, faeces, enteritis	C43
4 : · : —	4, 20, 22	A1, B3, C1	—	—	+	—	1	Hosp. 3, urine, cystitis	C47
4 : · : 1	4, 17, 20, 22	A1, B2, B3, C1	—	—	+	—	2	Hosp. 2, trachea, interstitial pneumonia	S 1/2
4 : 52L : —	2, 3, 4, 12, 15, 22, PO	A1, A4, B1, C1, S	—	—	—	—	1	Culture collection	A103

Table V

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroup O8

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
8 : 8L : 4	2, 3	A1	—	—	—	—	1	Culture collection	G/3404/41
8 : 8 : 20	7, 16, 17	A2, B2	—	—	—	—	1	Hosp. 1, faeces, enteritis	C112
8 : 8 : 20	5, 7, 16, 17	A2, B2	—	—	—	—	1	Hosp. 1, faeces, enteritis	C70
8 : 25B : 9	—	—	Phi, 17, K12	—	—	—	1	Culture collection	Bi 7575/41
8 : 27A : —	2, 3, 4, 5, 6, 7, 12, 14, 15	A1, A2, A4, B1	—	—	—	—	1	Culture collection	E 56 b
8 : 40A : 9	5, 7	A2	—	—	—	—	1	Culture collection	A 51 d
8 : 41A : 11	—	—	Phi, 17, K12	—	—	—	1	Culture collection	A 433 a
8 : 42A : —	—	—	—	—	—	—	1	Culture collection	A 295 b
8 : 43A : 11	2, 4, 12	A1, A4	Phi, 17, K12	—	—	—	1	Culture collection	A 195 a
8 : 44A : —	—	—	Phi, 17, K12	—	—	—	1	Culture collection	A 168 a
8 : 45A : 9	—	—	Phi, 17, K12	—	—	—	1	Culture collection	A 169 a
8 : 46A : —	3, 7	A1, A2	—	—	—	—	1	Culture collection	A 236 a
8 : 47A : 2	—	—	—	Phi, 17, K12	—	—	1	Culture collection	A 282 a
8 : 48A : 9	3	A1	Phi, 17, K12	—	—	—	1	Culture collection	A 290 a
8 : 49A : 21	2, 3, 4, 6	A1, A2	—	—	—	—	1	Culture collection	A 180 a
8 : 50A : —	—	—	17	—	—	—	1	Culture collection	PA 80 c
8 : : 20	—	—	—	—	—	—	1	Culture collection	H 330 b
8 : : 21	(3)	A1	—	—	—	—	1	Culture collection	V 11a/44
8(60) : : 19	(5), 7	A2	—	17	—	—	1	Hosp. 2, fomite	C 199
8(60) : : 19	(5), 7	A2	—	—	—	—	10	Hosp. 2, faeces, trachea, enteritis + pneumonia (9); air (1)	C 125 b
8(60) : : 19	—	—	—	—	—	—	2	Hosp. 2, fomite	C 196

serotype from Hospital 2, isolated from faeces, trachea and air of the wards, were identical in phage pattern, and colicinogenic and lysogenic properties.

Results for O18 strains are summarized in Table VI. Of the 38 strains* belonging to serogroup O18, 17 were isolated from the throat, trachea or faeces of patients suffering from bronchopneumonia, spastic bronchiolitis, interstitial pneumonitis or other respiratory diseases, 13 were obtained from the faeces of infants suffering from enteritis, and 6 were isolated from the faeces or throat secretion of infants suffering from both enteritis and respiratory disease. Strains O18 exhibited 15 different phage patterns: the joint occurrence of phages 4, 12, 13, 16, 17 and 18 in the phage pattern was characteristic. Typical phage groups were: A1, A4 and B2. Strains belonging to the same serotype exhibited phage patterns. None of the strains was colicinogenic while 31 were lysogenic, 21 caused haemolysis and all of them were PP negative. The strains collected in Hospital 1 and Hospital 2 were identical.

The strains of group O59 were first identified by their phage pattern which was identical with that of strain O59 supplied by the Culture Collection. Our supposition that these strains were identical with strain O59 of the Culture Collection in their antigenic structure, too, has been confirmed by serological tests performed at the International Escherichia Centre. Of the strains, 4 were isolated** from the faeces of the same premature infant and 2 were obtained from the air of the incubates in which the infant excreting the above 4 strains was kept. The pathologic role of the strain can be but supposed for the time being. According to the clinical diagnosis, the premature infant suffered from enteritis and interstitial pneumonia. To our knowledge, no pathogenic role in causing enteritis has yet been described for any of the strains belonging to serogroup O59. Phage pattern and phage group were identical in every strain. The characteristic phage group was A1, A2, A3, G1. The strains were colicinogenic except two isolated from air, and lysogenic except one also originating from air. None of the strains produced haemolysin. The strain obtained from the Culture Collection was PP negative while the other strains were PP positive. The results are shown in Table VII.

The results for strains belonging to serogroup O111 are shown in Table VIII. The strains could be divided into 8 phage patterns and 7 phage groups. The two most frequent phage patterns were: 8, 9 and 12, 21 (phage groups A3 and A3, B4). Twenty strains were colicinogenic and 11 were lysogenic. Strain with H antigen 25 as well as strains containing unknown H antigens were PP negative while the others (without H antigen and containing H2 antigen) were PP positive. One of the O111 strains was lysed also by the *Salmonella* phage "PO". The strains originating from the same hospital were iden-

* Of these, 24 strains were isolated by Dr. M. LAKATOS, in Debrecen, and 6 strains were isolated by Dr. F. BARON, in Kaposvár.

** Isolated by Dr. Z. BÉKÉSSY at the Budapest Public Health Station.

tical in their phage pattern, phage group, colicinogeny, lysogeny and PP reaction. The strains obtained outside the hospital were different in the above listed properties (Table VIII).

The strains belonging to serogroup O124 (Table IX) were derived from adult outpatients with enteritis or food poisoning. Identical numerals standing before "outpatient" in Table IX mean the same community (residence). The strains in this serogroup were fairly uniform in their phage pattern, so that colicinogeny provided a more adequate basis for their differentiation. Of the strains, 10 showed the same phage pattern (2, 3, 4, 6, 7), 12 belonged to phage group A1, A2, two strains were phage resistant, 7 colicinogenic and 4 lysogenic. Twelve strains were PP positive, none of them produced haemolysis. No correlation was found between phage pattern and serotype. The strains obtained from the same community were different in their serotype, phage pattern and colicinogeny, thus they had presumably originated from different sources. The strains isolated from the same family or from food poisoning were identical in phage pattern, phage group, colicinogeny, lysogeny and PP reaction.

Tables X and XI show the results obtained for various strains (O26, O55, O86, O112, O119, O125, O126, O127) of *E. coli* associated with infantile enteritis. Studying strains of different serotypes within the same serogroup, it has been found that the strains belonging to the same serotype are different in their phage sensitivity, colicinogenic and lysogenic property (in serogroups O26 and O55). The strains belonging to the other serogroups are of different serotypes and origin and differ also in phage pattern.

Table XII shows the strains of serogroups O1, O2 and O3. When isolated from the same source, strains O2 and O3 exhibited identical phage patterns.

Of the strains (O6) shown in Table XIII, those obtained from the same hospital during the same period exhibited identical serotypes and phage patterns. Two strains of the same serotype originating from the same hospital were lysed by *Salmonella* phage O.

Of the strains of serogroup O9 shown in Table XIV, some with different serotypes exhibited identical phage patterns.

Tables XV to XXI show strains of different serogroups and serotypes obtained from the Culture Collection and those of corresponding serogroups isolated from patients. In serogroups O21, O44 and O75, strains identical in serotype and phage pattern occurred in the same hospital. The strains of serogroup O115 isolated from the faeces of infants suffering from enteritis were different in phage pattern and colicinogenic property.

Summarizing the results, it may be stated that there is no correlation between the sensitivity to a certain phage and a certain serogroup or serotype. Nevertheless, some serogroups may be deduced from some phage patterns—e.g., serogroup O4 is characterized by phage group A1, B2, B3, C1, serogroup O18 by phage group A1, A4, B2, and serogroup O59 by phage group A1, A2, A3, G1.

Table VI

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroup O18

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
18ab : 76 : 14	7, 12, 24	A2, A4, D1	—	—	—	—	1	Culture collection	F10018/41
18ac : 77 : 7	4, 12, 16, 17	A1, A4, B2	—	—	—	—	1	Culture collection	3219/54
18ac : : : 5	12, 16, 17	A4, B2	—	Phi	+	—	1	Hosp. 3, faeces, enteritis	C38
18ac : : : 7	(12) (18) (24)	(A4, B2, D1)	—	Phi, 17	+	—	3	Hosp. 1, faeces, enteritis	C93
18ac : : : 7	4, 16, 17, 18	A4, B2	—	Phi, 17	—	—	1	Hosp. 1, throat, re- spiratory disease	C65
18ac : : : 7	(12) 16, 17, 18	(A4), B2	—	Phi, 17, K12	—	—	1	Hosp. 1, faeces, re- spiratory disease	C56
18ac : : : 7	4 (12) 13, 16, 17	A1, A4, B2	—	17	+	—	1	Hosp. 2, trachea, interstitial pneumonia	S4/2
18ac : : : 7	4(12)13, 16, 17	A1, A4, B2	—	Phi, 17	+	—	3	Hosp. 1, spastic bronchitis (1); Hosp. 2, trachea, interstitial pneumonia (2)	C7
18ac : : : 7	4, 13, 16, 17, 18	A1, A4, B2	—	Phi, 17, K12	—	—	1	Hosp. 1, faeces, enteritis + respiratory disease	C23
18ac : : : 7	4, (12), 16, 17, 18	A1, A4, B2	—	Phi, 17	—	—	1	Hosp. 1, faeces enteritis	C81
18ac : : : 7	4, (12), 16, 17, 18	A1, A4, B2	—	Phi, 17, K12	—	—	1	Hosp. 1, faeces, enteritis + respiratory disease	C99
18ac : : : 7	4, (12), 16, 17, 18	A1, A4, B2	—	Phi	—	—	1	Hosp. 1, throat, spastic bronchitis	C57
18ac : : : 7	4, (12), 13, 16, 17, 18	A1, A4, B2	—	17	—	—	1	Hosp. 1, throat, re- spiratory disease	C96
18ac : : : 7	4, (12), 13, 16, 17, (18)	A1, A4, B2	—	Phi, 17	+	—	1	Hosp. 2, trachea, in- terstitial pneumonia	S7/2

5*	18ac : : : 7	4, (12), 13, 16, 17, 18	A1, A4, B2	—	Phi, 17, K12	—	—	7	Hosp. 1, throat (2): enteritis; enteritis + respiratory disease; faeces (5): respiratory disease	C27
	18ac : : : ?	4, (12), 13, 16, 17, 18	A1, A4, B2	—	Phi, 17	+	—	1	Hosp. 1, faeces enter- itis	C115
	18ac* : : : —	(12)	(A4)	—	—	+	—	1	Hosp. 3, faeces enter- itis	C48
	18ac* : : : —	4, (12)	A1, (A4)	—	Phi	+	—	1	Hosp. 3, faeces, enter- itis	C52
	18ac* : : : —	4, (12), 18	A1, (A4), B2	—	Phi, 17	+	—	1	Hosp. 3, faeces, enteritis	C46
	18ac* : : : —	(12), (16), 17	(A4), B2	—	17	+	—	1	Hosp. 3, faeces, enteritis	C42
	18ac* : : : —	4, 16, 17	A1, B2	—	—	—	—	1	Hosp. 1, faeces, enter- itis + broncho- pneumonia	C107
	18ac* : : : —	4, 12, 16, 17	A1, A4, B2	—	Phi	—	—	2	Hosp. 1, faeces, enter- itis + bronchopneu- monia (1)	C1
	18ac* : : : —	4, 12, 16, 17	A1, A4, B2	—	—	—	—	1	Hosp. 1, faeces, enter- itis + bronchopneu- monia	C30
	18ac* : : : —	4, 13, 16, 17	A1, A4, B2	—	Phi, 17	+	—	1	Hosp. 2, trachea, interstitial pneumonia	S18a1
	18ac* : : : ?	4, (12), 13, 16, 17	A1, A4, B2	—	—	—	—	1	Hosp. 1, faeces, respiratory disease	C104
	18ac** : : : —	7, 12	A2, A4	—	17	—	—	2	Hosp. 4, faeces, enter- itis (2)	C585

* No cross reaction with O17 and O106 sera

** Cross reaction with O126 : K71 serum

Table VII

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of E. coli serogroup O59

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
59 : . : 19	1, 2, 3, (6), 7, 8, 9, 10, 11, 28	A1, A2, A3, G1	Phi	K12	—	+	1	Hosp. 2, faeces, enteritis + respiratory disease	189a
59 : . : 19	1, 2, 3, (6), 7, 8, 9, 10, 11, 28	A1, A2, A3, G1	Phi	Phi	—	+	1	Hosp. 2, faeces, en-ritis + respiratory disease	189b
59 : . : 19	1, 2, 3, 6, 7, 8, 9, 10, 11, 28	A1, A2, A3, G1	Phi, K12	Phi, K12	—	+	1	Hosp. 2, faeces, enteritis + respiratory disease	126a
59 : . : 19	1, 2, 3, (6), 7, 8, 9, 10, 11, 28	A1, A2, A3, G1	Phi, K12	K12	—	+	1	Hosp. 2, faeces, enteritis + respiratory disease	126b
59 : . : 19	1, 2, 3, (6), 7, 8, 9, 10, 11, 28	A1, A2, A3, G1	Phi, 17, K12	K12	—	—	1	Culture collection	F9095/41
59 : . : 19	1, 2, 3, (6), 7, 8, 9, 10, 11, 28	A1, A2, A3, G1	—	Phi	—	+	1	Hosp. 2, air	198
59 : . : 19	1, 2, 3, (6), 7, 8, 9, 10, 11, 28	A1, A2, A3, G1	—	—	—	+	1	Hosp. 2, air	195

Table VIII

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of E. coli serogroup O111

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
111 : 58B : —	1, 8, 9, 10, 21	A1, A3, B4 B4	Phi, 17 K12	—	—	+	1	Culture collection	Stoke W
111 : 58B : 2			—	Phi, 17, K12	+	+	1	Outpatient unit faeces, enteritis	C346
111 : 58B : 2	21	B4	—	17	+	+	1	Outpatient unit faeces, enteritis	C348
111 : 58B : 2	8, 9	A3	—	Phi, 17, K12	—	+	4	Hosp., faeces, enteritis (3); Outpatient faeces, enteritis (1)	C9
111 : 58B : 2	10, 21	A3, B4	—	17	—	+	1	Hosp. 5, faeces, enteritis	C577
111 : 58B : 2	8, 9, 10, 12, 28	A3, A4, C1	—	17	—	+	1	Hosp. 5, faeces, enteritis	C578
111 : 58B : 25	12	A4	Phi, K12	17	+	—	1	Outpatient unit faeces, enteritis	C351
111 : 58B : 25	(12)	(A4)	Phi, K12	—	—	—	1	Outpatient unit faeces, enteritis	C58
111 : 58B : 25	12, PO	A4, S	Phi, K12	—	—	—	1	Outpatient unit faeces, enteritis	C63
111 : 58B : 25	12, 21	A4, B4	Phi, K12	—	—	—	1	Outpatient unit faeces, enteritis	C116
111 : 58B : .	12, 21	A4, B4	Phi, 17, K12	—	—	—	15	Hosp. G. D. R. faeces, enteritis	C223
111 : 58B : .	12, 21	A4, B4	—	—	—	—	1	Hosp. G. D. R. faeces, enteritis	C235
111 : 58B : .	—	—	—	Phi, 17, K12	—	+	2	Hosp. 6, faeces, enteritis	C579

Table IX

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of E. coli serogroup O124

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
124 : 72? : —	2, 3, 6, 7	A1, A2	Phi, K12	—	—	+	1	Outpatient unit 1, faeces, enteritis	C359
124 : 72? : —	2, 3, (4), 6, (7)	A1, A2	—	17	—	—	1	Outpatient unit 4, faeces, enteritis	C455
124 : 72? : —	2, 3, (4), (6), (7)	A1, A2	—	—	—	—	1	Outpatient unit 7, faeces, enteritis	C646
124 : 72? : —	2, 3, 4, 6, (7), 17	A1, A2, B2	—	—	—	+	1	Outpatient unit 2, faeces, enteritis	C368
124 : 72? : —	2, 3, 4, 6, (7), 12, 14, (15), (23)	A1, A2, A3, B1, D1	—	—	—	—	1	Outpatient unit 2, faeces, enteritis	C367
124 : 72? : —	—	—	Phi, 17, K12	17	—	+	1	Outpatient unit 1, faeces, enteritis	C377
124 : 72? : 30	2, 3, 6, 7	A1, A2	Phi, 17, K12	17	—	—	1	Outpatient unit 3, faeces, enteritis	C468
124 : 72? : 30	2, 3, (4), (6), (7)	A1, A2	Phi K12	17	—	+	1	Outpatient unit 3, faeces, enteritis	C420
124 : 72? : 30	2, 3, 4, 6, (7)	A1, A2	Phi K12	—	—	+	2	Outpatient unit 1, faeces, enteritis. Outpatient unit 6, faeces, enteritis	C360
124 : 72? : 30	2, 3, 4, 6, 7	A1, A2	—	—	—	+	3	Outpatient unit 4, faeces, food-poisoning (2). Outpatient unit 5, faeces, enteritis (1)	C657
124 : 72? : 30	2, 3, 4, 6, (7)	A1, A2	—	—	—	—	1	Outpatient unit 1, faeces, enteritis	C366
124 : 72? : 30	—	—	—	—	—	+	1	Outpatient unit 2, faeces, enteritis	C371
124 : 72B : 32?	2, 3, 4	A1	Phi	—	—	+	1	Culture collection	Parádsasvár 916
124 : 72B : 32?	2, 3, (4), 6, 7	A1, A2	—	—	—	+	1	Culture collection	227

Table X

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP-reaction of different *E. coli* serogroups associated with infantile enteritis (O26, O55, O86)

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
26 : 60B : —	1, 8, 27	A1, A3, F2	—	—	—	—	1	Outpatient unit 1, faeces, enteritis	C115
26 : 60B : —	2, 8, 16, 27	A1, A3, B2, F2	—	—	+	—	1	Outpatient unit 2, faeces, enteritis	C340
26 : 60B : —	1, 2, 6, 8, 9, 12, 20, 21, 27	A1, A2, A3, A4, B3, B4, F2	—	—	—	—	1	Culture collection	F 41
26 : 60B : 11	8, 15, 20, 27	A3, B1, B3, F2	Phi, 17, K12	—	—	—	1	Culture collection	H311 b
26 : 60B : 46	2, 12, 15	A1, A4, B1	—	—	—	—	1	Culture collection	5306/56
55 : 59B : —	7	A2	Phi K12	—	—	+	1	Culture collection	Su3912/41
55 : 59B : —	2, 8, 9, 12, 28	A1, A3, A4, G1	K12	17	—	+	1	Hosp. 4, faeces, enteritis	C538
55 : 59B : 6	20	B3	—	—	—	—	1	Culture collection	Aberdeen 1064
55 : 59B : 6	19, 20	B2, B3	—	Phi, 17	+	—	1	Outpatient unit 4, faeces, enteritis	C354
55 : 59B : 7	2, 20	A1, B3	Phi, 17, K12	—	—	—	1	Outpatient unit 3, faeces, enteritis	C122
55 : 59B : 7	1, 2, 3, 6	A1, A2	Phi, 17, K12	—	—	—	1	Hosp. 4, faeces, enteritis	C630
55 : 59B : .	2, 3	A1	Phi, 17, K12	—	—	—	1	Hosp. 4, faeces, enteritis	C586
55 : 59B : .	—	—	Phi, 17	—	—	—	1	Culture collection	972
86 : 61B : —	—	—	—	—	—	—	1	Culture collection	E990/1957
86 : 61B : —	—	—	—	—	—	—	1	Culture collection	E990/1962
86 : 61B : 34	—	—	—	—	—	—	1	Culture collection	BD12665
86 : 62L : 2	23	D1	—	—	—	+	1	Culture collection	F 1961
86ab : 64B : 36	14, (23), 24	B1, D1	—	—	—	—	1	Culture collection	5017/53
86 : . : 10	12	A4	K12	17	+	—	1	Outpatient, faeces, enteritis	C337
86 : . : 10	12	A4	—	—	+	—	1	Outpatient, faeces enteritis	C339
86 : — : 25	(6)	(A2)	—	—	—	—	1	Culture collection	H 35
86 : . : 47	12	A4	—	Phi	—	—	1	Culture collection	1755/58

Table XI

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of different E. coli serogroups associated with infantile enteritis (0112, 0119, 0125, 0126, 0127, 0128)

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
112ac : 66B : .	16, 17	B2	—	—	—	—	1	Culture collection	Guanabara M194/957 1411/50
112 : 68B : 18	17	B2	—	—	—	—	1	Culture collection	
119 : 69B : 6	(2, 6), 17	(A1, A2), B2	—	—	+	—	1	Culture collection	537/52 W 34
119 : 69B : 27	—	—	—	—	+	—	1	Culture collection	
125 : 11(L) : —	—	—	—	—	—	—	1	Culture collection	F 103
125ab : 70(B) : 7?	12	A4	—	Phi, 17, K12	—	—	1	Outpatient unit 1, faeces, enteritis	
125ab : 70(B) : 10	12, 17	A4, B2	—	—	—	—	1	Outpatient unit 2, faeces, enteritis	C126 Canioni (2745)53
125 : 70(B) : 19	—	—	—	—	—	—	1	Culture collection	
125 : 70(B) : 32	1, 2, 6, 8, 10	A1, A2, A3	—	—	—	—	1	Outpatient unit 3, faeces, enteritis	C117
126 : 71B : 2	28	G1	—	—	—	+	1	Culture collection	E611 6021/50
127 : 63B : —	—	—	—	—	—	—	1	Culture collection	4932/53 1957, 1962
127 : 63? : 21	2, 3, 6, 12	A1, A2, A4	—	—	—	—	1	Outpatient, faeces, enteritis	
128 : 67(B) : 2	1, 8, 20, 24	A1, A3, B3, D1	—	—	—	+	1	Culture collection	Cigleris 56/54
128 : 67(B) : 12	20	B3	—	—	—	—	1	Outpatient unit 1, faeces, enteritis	
128 : 67(B) : 35	1, 8, 9, 14, 20, 24	A1, A3, B1, B3, D1	—	—	+	—	1	Outpatient unit 2, faeces, enteritis	C125 C338

Table XII

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroups O1, O2, O3

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
1 : 1L : 7 1 : 51L : — 1 : · : 7	3 4, 15 15, 26	A1 A1, B1 B1, F1	Phi, 17, K12 — Phi, 17, K12	— — Phi, 17, K12	— — —	— — —	1 1 1	Culture collection Culture collection Hosp. 1, faeces, enteritis	U 5/41 A183a C76
2 : 1L : 4 2 : 1L : 6 2 : 1 : 4	3, 4 4, 12 —	A1 A1, A4 —	Phi, 17, K12 Phi, 17, K12 Phi, 17, K12	— — —	— — —	— — —	1 1 2	Culture collection Culture collection Hosp. 2, faeces, enteritis, enteritis + respiratory disease (1); enteritis (1)	U 9/41 A20a C 33b
2 : 2L : 1 2 : 56 : 7 2 : · : 4	4, 12 (15) (12) 12, 23	A1, A4, (B1) (A4) A4, D1	— Phi, 17, K12 Phi, 17, K12	— — Phi, 17, K12	— — Phi, 17, K12	— — —	1 1 1	Culture collection Culture collection Hosp. 2, faeces, enteritis	Su 1242 H17b C147a
2 : · : 4	12, 13	A4, D1	Phi, 17, K12	—	—	—	1	Hosp. 2, faeces, enteritis	C41a*
2 : · : 4	12, 15, 23	A4, B1, D1	Phi, 17, K12	—	—	—	1	Hosp. 2, faeces, enteritis	C127b*
2 : · : 8	12, 15	A4, B1	—	—	—	—	1	Culture collection	AP320c
3 : 2ab(L) : 2 3 : · : 44 3 (68, 115, 119) : · : · : —	(2), 3, 23 4 2, 3, PO	A1, D1 A1 A1, S	Phi, 17, K12 Phi, 17, K12 —	— — —	— — —	— — —	1 1 2	Culture collection Culture collection Hosp. 7, faeces, enteritis	U 14/41 781/55 C123

* Same box.

Table XIII

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of E. coli serogroup O6

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
6 : 2ac(L) : 1	4, 15	A1, B1	—	—	+	—	1	Culture collection	Bi 7458/41
6 : 13 : 1	4	A1	—	—	—	—	2	Hosp. 1, faeces, enteritis	C 25
6 : 13 : 1	4	A1	—	Phi	—	—	1	Hosp. 1, throat, enteritis	C 26
6 : 13 : 1	4, 5	A1, A2	—	—	+	—	2	Hosp. 1, throat, enteritis (1)	
								Hosp. 1, faeces, enteritis (1)	C 103
6 : 13(L) : 1	4, (7)	A1, (A2)	Phi, K12	—	—	—	1	Culture collection	Su 4344/41
6 : 13(L) : 49	12	A4	Phi, 17, K12	—	—	—	1	Culture collection	2147/59
6 : 15(L) : 16	2, 3, 4, 6, 12	A1, A2, A4	—	—	—	—	1	Culture collection	F8316/41
	14, 17, 28	B1, B2, G1							
6 : 53(L) : —	4, 15 (17)	A1, B1, (B2)	17	—	+	—	1	Culture collection	PA 236
6 : 54(L) : 10	(4), 14	(A1), B1	—	—	—	—	1	Culture collection	A 12 b
6 : . : 10	PO	S	Phi, 17, K12	—	+	—	1	Hosp. 3, faeces, enteritis	C 257
6 : . : 10	12, 17, PO	A4, B2, S	Phi, 17, K12	—	+	—	1	Hosp. 3, faeces, enteritis	C 303

Table XIV

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of E. coli serogroup O9

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemolysis	PP	No. of strains	Source	Type strain
			pattern						
9 : 9L : 12	—	—	Phi, 17, K12	—	—	—	1	Culture collection	Bi 316/42
9a : 26A : —	—	—	—	—	—	—	1	Culture collection	Bi 449/42
9a,b : 28A : —	5	A2	—	—	—	—	1	Culture collection	K 14a
9 : 29A : —	(2), 3, 4, (5), (7), 12	A1, A2, A4	Phi, 17, K12	—	+	—	1	Culture collection	Bi 161/42
9 : 30A : 1	2, 3, 5, 6, 7	A1, A2	Phi, 17, K12	—	+	—	1	Culture collection	E 69
9 : 31A : —	2	A1	—	—	—	—	1	Culture collection	Su 3973/41
9 : 32A : 10	2, 3, 4, 5, 7, 12	A1, A2, A4	Phi, 17, K12	—	+	—	1	Culture collection	H 36
9 : 33A : —	(3), 4, (5, 6), 7, (11), 12	A1, A2, (A3), A4	—	—	—	—	1	Culture collection	AP 289
9 : 34A : —	—	—	Phi, 17, K12	—	—	—	1	Culture collection	E 75
9 : 35A : —	2, 3, 4, 5, 12	A1, A2, A4	—	—	—	—	1	Culture collection	A 140a
9 : 36A : 19	4, (7)	A1, (A2)	—	—	—	—	1	Culture collection	A 198a
9 : 37A : —	2, 3, 9, 12, 15, 23, 24	A1, A3, A4, B1, D1	—	—	—	—	1	Culture collection	A 84a
9 : 38A : —	—	—	—	—	—	—	1	Culture collection	A 262a
9 : 39A : 9	2, 3, 5, 6, 7, 12	A1, A2, A4	—	—	—	—	1	Culture collection	A 121a
9 : 55A : —	—	—	—	—	—	—	1	Culture collection	N 24c
9 : 57B : 32	—	—	Phi, 17, K12	—	—	—	1	Culture collection	H 509d
9 : 66? : 10	2, 3	A1	—	17	—	+	1	Outpatient, faeces, enteritis	C 460
9 : : 19	2, 3, 4, 5, 6, 7, 12	A1, A2, A4	Phi, 17, K12	—	—	—	1	Culture collection	A 18d

Table XV

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroups 05, 07, 010, 011, 012, 013, 014, 015, 016, 017, 019, 020

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
5 : 4L : 4	(2), (3), 4, 12	A1, A4	—	—	—	—	1	Culture collection	U1/41
7 : 1L : —	4	A1	Phi, 17, K12	—	—	—	1	Culture collection	Bi 7509/41
7 : 7L : 4	12	A4	—	—	—	—	1	Culture collection	Pus 3432/41
10 : 5L : 4	(2), (3), 4, 6, 12, 16, 17, PO	A1, A2, A4, B2, S	—	—	—	—	1	Culture collection	Bi 8337/41
11 : 10L : 10	12, (17), 22, PO	A4, (B2), C1, S	—	—	—	—	1	Culture collection	Bi 623/42
11 : . : 33	12, 22	A4, C1	—	—	—	—	1	Culture collection	K 181
12 : 5L : —	2, 3, 4, 6, 12, 16, (17), (20)	A1, A2, A4, B2 (B3)	—	—	—	—	1	Culture collection	Bi 626/42
13 : 11L : 11	—	—	—	—	—	—	1	Culture collection	Su 4321/41
14 : 7L : —	15, (16), 17	B1, B2	—	—	—	—	1	Culture collection	Su 4411/41
15 : 14L : 4	4, (12)	A1, (A4)	Phi, 17, K12	—	—	—	1	Culture collection	F 7902/41
15 : . : 17	(4), 15	(A1), B1	—	—	—	—	1	Culture collection	P 12 b
15 : . : 27	(14), (PO)	(B1), (S)	—	—	—	—	1	Culture collection	K 50
16 : 1L : —	(4)	(A1)	Phi, 17, K12	—	—	—	1	Culture collection	F 11119/41
16 : . : 48	12	A4	—	—	—	—	1	Culture collection	P 4
17 : 16L : 18	9, 12, (28)	A3, A4, (G1)	—	—	—	—	1	Culture collection	K 12a
19b : . : 7	—	—	—	Phi	—	—	1	Culture collection	F 8188/41
19 : . : 29	—	—	—	—	—	—	1	Culture collection	N 282
20 : 17L : —	2, 3, (4), 6, 7, 12, (14), 23, 24	A1, A2, A4, (B1), D1	—	Phi, 17, K12	—	—	1	Culture collection	P 7 a
20ab : 83B : 26	—	—	—	—	—	—	1	Culture collection	134/51
20ab : 84B : 26	7	A2	Phi, 17, K12	—	—	—	1	Culture collection	2292/55
20 : 67 : 17	12	A4	—	—	—	—	1	Hosp. 4, faeces, enteritis	C 651

Table XVI

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroups O21, O22, O23, O24, O25, O27, O28, O29, O30, O32, O33, O34, O35, O36

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
21 : 20L : — 21 : 20 : 4	4, (15) 4	A1, (B1) A1	— —	— —	— —	— —	1 1	Culture collection Hosp. 2, throat, anaemia	E 19a C 213
21 : 20 : 4	4, 20	A1, B3	—	—	—	—	1	Hosp. 2, nose, anaemia	C 210
21 : . : 19	4	A1	—	—	—	—	3	Hosp. 2, faeces, re- spiratory disease (1) Hosp. 2, air (2)	C 151a
22 : 13L : 1	7, (15)	A2, (B1)	—	K12	+	—	1	Culture collection	E 14a
22 : 24L : 31	(2), 3, 4, (6)	A1, (A2)	—	—	—	—	1	Culture collection	H 45
23 : 18L : 15	4	A1	—	—	—	—	1	Culture collection	E 39a
23 : 22L : 15	2, 3, 4, 6, 7, 14, 15, 23, 24	A1, A2, B1, D1	—	—	—	—	1	Culture collection	H 67
24 : . : —	—	—	Phi, 17, K12	—	—	—	1	Culture collection	E 41a
25 : 19L : 12	4, 12, 14, 24	A1, A4, B1, D1	—	—	—	—	1	Culture collection	E 47a
25 : 23L : 1	4	A1	—	K12	—	—	1	Culture collection	H 54
25 : . : ?	4	A1	—	—	—	—	1	Hosp. 1, throat, enteritis	C 108
27 : . : —	—	—	—	—	—	—	1	Culture collection	F 9884/41
27 : . : 20	4	A1	—	—	—	—	1	Hosp. 1, faeces, enteritis	C 74
28 : 73B : .	2, 3, (23)	A1, (D1)	—	—	+	—	1	Culture collection	Katwijk
28 : . : —	(2), 3, 6, PO	A1, A2, S	Phi, 17, K12	—	—	—	1	Culture collection	K 1a
29 : . : 10	2, 3, (6)	A1, (A2)	Phi, 17, K12	—	—	—	1	Culture collection	Su 4338/41
30 : . : —	2, 3, 6, 7	A1, A2	Phi, 17, K12	—	+	—	1	Culture collection	P 2a
32 : 87B : 45	2, 3, 6, 7, 17	A1, A2, B2	—	Phi, 17, K12	—	—	1	Culture collection	145
32 : . : 19	—	—	—	—	—	—	1	Culture collection	P 6a
33 : . : —	7, 17	A2, B2	—	—	—	—	1	Culture collection	E 40
34 : . : 10	—	—	—	—	—	—	1	Culture collection	H 304
35 : . : 10	4	A1	Phi, 17, K12	—	—	—	1	Culture collection	E 77a
36 : . : 9	(8), 9, (12), 22	A3, A4, C1	Phi, 17,-K12	—	—	—	1	Culture collection	H 502a

Table XVII

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of E. coli serogroups 037, 038, 039, 040, 041, 042, 043, 044, 045, 046, 048, 049, 050, 051, 052

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
37 : : 10	PO	S	Phi, 17, K12	Phi	—	—	1	Culture collection	H 510c
38 : : 26	3, (7), 20, PO	A1, (A2), B3, S	—	—	—	—	1	Culture collection	F 11621/41
38 : : 30	(7)	(A2)	Phi, 17, K12	17	—	—	1	Culture collection	N 157
39 : : —	(12)	(A4)	—	—	+	—	1	Culture collection	H 7
40 : : 4	12, (17)	A4, (B2)	—	—	—	—	1	Culture collection	H 316
41 : : 40	2, 3	A1	—	—	—	—	1	Culture collection	H 710c
42 : : 37	2, 3, 6, 7, (16), 17	A1, A2, B2	Phi, 17, K12	—	—	—	1	Culture collection	P 11a
43 : : 2	12, 20	A4, B3	Phi, 17, K12	—	—	—	1	Culture collection	Bi 7455/41
44 : 74L : 18	23	D1	K12	—	—	—	1	Hosp. 3, faeces, enteritis	C 36
44 : 74L : 18	23	D1	K12	Phi	—	—	1	Hosp. 3, faeces, enteritis	C 44
44 : 74L : 18	8, 9	A3	Phi, 17, K12	—	+	—	1	Culture collection	H 702c
44 : 74L : 18	—	—	K12	—	—	—	1	Hosp. 3, faeces, enteritis	C 37
44 : :	4, 12, 17	A1, A4, B2	—	—	—	—	1	Culture collection	F 17
45 : : 10	(2), (3), 6, (12), (20)	(A1), A2, (A4), (B3)	Phi, 17, K12	—	+	—	1	Culture collection	H 61
45 : : 23	—	—	—	—	—	—	1	Culture collection	K 42
46 : : 16	2, 3, (6), 8, 9, 10, 11, 16, 17, (28)	(A1), (A2), A3, B2, G1	—	—	—	—	1	Culture collection	P 1c
48 : : —	2, (3), 6, 7	A1, A2	Phi, 17, K12	—	—	—	1	Culture collection	U 8/41
49 : : 12	(2), 3, (6), 12, (PO)	A1, (A2), A4, (S)	—	—	—	—	1	Culture collection	U 12/41
50 : : —	4, 7, (24)	A1, A2, (D1)	Phi, 17, K12	—	+	—	1	Culture collection	U 18/41
51 : : 24	4, 7	A1, A2	—	—	—	—	1	Culture collection	U 19/41
52 : : 10	(3), 4, (17), PO	A1, (B2), S	—	—	—	—	1	Culture collection	U 20/41
52ab : : 45	4, 8, (9), 12	A1, A3, A4	—	—	—	—	1	Culture collection	4106/54

Table XVIII

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroups 053, 054, 056, 057, 058, 060, 061, 062, 063, 064, 065, 066, 068, 069, 070, 071, 073, 074, 075

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemolysis	PP	No. of strains	Source	Type strain
			pattern						
53 : : 3	—	—	—	—	—	—	1	Culture collection	Bi 7327/41
54 : : 2	1, 2, 6, 7, 8, 9, 28	A1, A2, A3, G1	—	—	—	—	1	Culture collection	Su 3972/41
56 : : —	—	—	—	—	—	—	1	Culture collection	Su 3684/41
57 : : —	2, 3, (6), 7, 12, 15	A1, A2, A4	Phi, 17, K12	—	—	—	1	Culture collection	F 8198/41
58 : : —	—	—	—	—	—	—	1	Culture collection	F 8962/41
60 : : —	2, 3, (6), 12, 15	A1, (A2), A4, B1	—	—	—	—	1	Culture collection	F 10167a/41
61 : : 19	1, (6), 8, 9, 17	A1, (A2), A3, B2	Phi, 17, K12	—	—	—	1	Culture collection	F 10167b/41
62 : : 30	2, 3, 6, 12, PO	A1, A2, A4, S	Phi, 17, K12	—	+	—	1	Culture collection	F 10524/41
63 : : —	—	—	Phi, 17, K12	K12	+	—	1	Culture collection	F 10598/41
64 : : —	(7)	(A2)	—	—	—	—	1	Culture collection	K 6 b
65 : : —	2, 3	A1	(Phi), (K12)	—	—	—	1	Culture collection	K 11 a
66 : : 25	4, 12, 15, 24	A1, A4, B1, D1	—	—	—	—	1	Culture collection	P 1 a
68 : : 4	16, (17)	B2	—	Phi	—	—	1	Culture collection	P 7 d
69 : : 38	2, 3, 6	A1, A2	Phi, 17, K12	—	—	—	1	Culture collection	P 9 b
70 : : 38	(12)	(A4)	Phi, 17, K12	—	—	—	1	Culture collection	P 9 c
70 : : 42	17	B2	—	—	—	—	1	Culture collection	P 9 c
71 : : 12	(4), 12, (17)	(A1), A4, (B2)	Phi, 17, K12	—	+	—	1	Culture collection	P 10 a
73 : : 31	12, 15, 16, 17, 24	A4, B1, B2, D1	—	—	—	—	1	Culture collection	P 12 a
74 : : 39	2, 3, (6), 16, 17, PO	A1, (A2), B2, S	—	—	—	—	1	Culture collection	E 3 a
75 : : —	12	A4	—	—	+	—	1	Hosp. 2, trachea, interstitial pneumonia	S 19 a1
75 : : —	12	A4	K12	—	+	—	1	Hosp. 2, faeces, interstitial pneumonia	S 19 b1
75 : : 5	—	—	(Phi), (17), (K12)	—	+	—	1	Culture collection	E 3 b
75 : : 5	—	—	—	—	—	—	2	Hosp. 2, trachea, interstitial pneumonia (1); Hosp. 2, urine, interstitial pneumonia (1)	S 1/1

Table XIX

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroups 076, 077, 078, 079, 080, 081, 082, 083, 084, 085, 087, 088, 089, 090, 091, 092, 093, 095, 096, 097, 098, 099

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemolysis	PP	No. of strains	Source	Type strain
			pattern						
76 : : 8	(6)	(A2)	—	—	—	—	1	Culture collection	E 5 d
77 : : —	(4)	(A1)	Phi, 17, K12	—	—	—	1	Culture collection	E 10
78 : : —	4	A1	Phi, (17), K12	—	—	—	1	Culture collection	E 38
78 : 80B : —	4, (12)	A1, (A4)	Phi, 17, K12	—	—	—	1	Culture collection	E 38
79 : : 40	3	A1	Phi, 17, K12	—	—	—	1	Culture collection	E 49
80 : : 26	7	A2	—	—	—	—	1	Culture collection	E 71
81 : : —	1, 2, 3, 8, (9), (10), 23	A1, A3, D1	Phi, 17, K12	—	—	—	1	Culture collection	H 5
82 : : —	8, 9, 11, 14, 15, 28	A3, B1, G1	—	—	+	—	1	Culture collection	H 14
83 : : 31	(2), (3), 7	(A1), A2	—	—	+	—	1	Culture collection	H 17a
84 : : 21	2, 3, 7, 16, 17	A1, A2, B2	—	—	—	—	1	Culture collection	H 19
85 : : 1	12	A4	(Phi), (K12)	—	—	—	1	Culture collection	H 23
87 : : 12	2, (3), 7, PO	A1, A2, S	—	—	—	—	1	Culture collection	H 40
88 : : 25	4, 12	A1, A4	—	—	—	—	1	Culture collection	H 53
89 : : 16	1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 14, 15, 16, (17), 24, 28	A1, A2, A3, B1, B2, D1, G1	—	—	—	—	1	Culture collection	H 68
90 : : —	2, 3, 8, 9, 11, 12, 14, 15, 23, 24	A1, A3, A4, B1, D1	—	—	—	—	1	Culture collection	H 77
91 : : —	1, (6), 28	A1, (A2), G1	Phi, (17), K12	—	+	—	1	Culture collection	H 307b
92 : : 33	1, (4), (8), 9, 12	A1, A3, A4	—	—	—	—	1	Culture collection	H 308a
93 : : 8	(4), (5)	(A1), (A2)	Phi, 17, K12	—	—	—	1	Culture collection	H 308b
95 : : —	12, 15	A4, B1	—	—	—	—	1	Culture collection	H 311a
96 : : 19	2, 3, (9)	A1, (A3)	—	—	—	—	1	Culture collection	H 319
97 : : —	(1), 4, 8, 12, (15), 28	A1, A3, A4, (B1), G1	Phi, 17, K12	—	—	—	1	Culture collection	H 320a
98 : : 8	(1), 2, 3, (6), 8, (9), 10, 11	A1, A2, A3	—	—	—	—	1	Culture collection	H 501d
99 : : 33	12, (17)	A4, (B2)	—	—	—	—	1	Culture collection	H 504 e

Table XX

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroups O100, O101, O102, O103, O104, O105, O106, O107, O108, O109, O110, O113, O114, O115, O116, O117, O118, O120, O121, O123

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemolysis	PP	No. of strains	Source	Type strain
			pattern						
100 : : 2	(2), 7	(A1), A2	—	—	—	—	1	Culture collection	H 509a
101 : : 33	PO	S	—	—	—	—	1	Culture collection	H 510a
102 : : 8	12, (17)	A4, (B2)	—	—	—	—	1	Culture collection	H 511
103 : : 8	12, (15)	A4, (B1)	Phi, 17, K12	17	—	—	1	Culture collection	H 515b
104 : : 12	(2), 3, (6), (7)	A1, (A2)	—	—	—	—	1	Culture collection	H 519
105 : : 8	1, 2, 3, (6), 7, 28	A1, A2, G1	Phi, 17, K12	—	—	—	1	Culture collection	H 520b
106 : : 33	12, 24	A4, D1	Phi, 17, K12	—	—	—	1	Culture collection	H 521a
107 : : 27	2, 3, 4, (6), (23)	A1, (A2), (D1)	—	—	—	—	1	Culture collection	H 705
108 : : 10	—	—	Phi, 17, K12	—	—	—	1	Culture collection	H 708b
109 : : 19	4, (7), (12), (14), 15, 23, (24)	A1, (A2), (A4), (B1), D1	—	—	—	—	1	Culture collection	H 709c
110 : : 39	2, 3, 9, (15), 16, 17, 23, 24	A1, A3, B2, D1, B1	—	—	—	—	1	Culture collection	H 711c
113 : 75B : 21	2, (3), 4, (6), 7, 12, 15, (16), (17)	A1, A2, A4, B1, (B2)	—	—	—	—	1	Culture collection	6182/50 (W 32)
114 : : 32	—	—	Phi, 17, K12	—	—	—	1	Culture collection	W 26
115 : : —	8	A3	Phi, K12	—	—	—	1	Hosp. 1, faeces, enteritis	C 106
115 : : —	1, 8, 9, 10	A1, A3	—	—	—	—	1	Hosp. 1, faeces, enteritis	C 118b
115 : : 18	2, 3, 7	A1, A2	Phi, 17, K12	—	—	—	1	Culture collection	W 27
115 : : 32	4	A1	—	—	—	—	1	Hosp. 1, faeces, enteritis	C 82
116 : : 10	2, (3), (6)	A1, (A2)	Phi, 17, K12	—	—	—	1	Culture collection	W 28
117 : : 4	—	—	—	—	—	—	1	Culture collection	W 30
118 : : —	(4)	(A1)	Phi, 17, K12	—	—	—	1	Culture collection	W 31
120 : : 6	(2), 4, 7	A1, A2	Phi, 17, K12	—	—	—	1	Culture collection	W 35
121 : : 10	(2), 3, (6), 7	A1, A2	—	—	+	—	1	Culture collection	W 39
123 : : 16	—	—	Phi, 17, K12	—	—	—	1	Culture collection	W 43

Table XXI

Phage pattern, phage group, colicinogeny, lysogeny, haemolysis and PP reaction of *E. coli* serogroups 0129, 0130, 0131, 0132, 0133, 0134, 0135, 0136, 0137, 0138, 0139, 0140, 0141, 0142, 0143, 0144, 0145, 0146

Antigenic structure	Phage pattern	Phage group	Colicin	Lysogen	Haemo-lysis	PP	No. of strains	Source	Type strain
			pattern						
119 : · B : 11	1, 3, 4, 12, 15	A1, A4, B1	—	—	—	—	1	Culture collection	178/54
130 : · · B9	(4), 7	A1, A2	—	—	—	—	1	Culture collection	4866/53
131 : · · ·	(2), 6	(A1), A2	Phi, (17), K12	—	—	—	1	Culture collection	S 239
131 : · · 26	(6)	(A2)	Phi, (17), K12	—	—	—	1	Culture collection	S 239
132 : · · ·	3, 4, 12	A1, A4	—	(K12)	—	—	1	Culture collection	N 87
132 : · · 28	(3), 4, 12	A1, A4	—	—	—	—	1	Culture collection	N 87
133 : · · ·	2,3, 4, (6), (7)	A1, (A2)	—	—	—	—	1	Culture collection	N 282
134 : · · ·	(2)	(A1)	—	—	—	—	1	Culture collection	4370/53
134 : · · 35	—	—	—	—	—	—	1	Culture collection	4370/53
135 : · · ·	(2), 3, 8, 9, (10), 12, 14, 15, (20), (27)	A1, A3, A4, B1, B3, F2	—	—	—	—	1	Culture collection	Coli Pécs
136 : · · ·	(2), 3, (4), (23)	A1, (D1)	—	—	—	—	1	Culture collection	1111/55
136 : 78B : —	2, 3, (4)	A1	—	—	—	—	1	Culture collection	1111/55
137 : · · ·	—	—	Phi, (K12)	—	—	—	1	Culture collection	RVC 1787
137 : 79L : ·	—	—	Phi, (17), (K12)	—	—	—	1	Culture collection	RVC 1787
137 : 79L : 41	—	—	Phi, (17), (K12)	Phi	—	—	1	Culture collection	RVC 1787
138 : · · ·	—	—	—	—	—	—	1	Culture collection	CDC 62/57
138 : 81B : ·	—	—	—	—	+	—	1	Culture collection	62/57
139 : · · ·	—	—	Phi, (17), (K12)	—	—	—	1	Culture collection	CDC 63/57
139 : 82B : 1	6, 7	A2	Phi, (17), (K12)	—	—	—	1	Culture collection	63/57
140 : · · ·	1, 2, 3, 6, 7, 8, 9, 17	A1, A2, A3, B2	—	—	—	—	1	Culture collection	CDC 149/51
140 : · · 43	1, (2), 3, 6, 7, 8, (9)	A1, A2, A3	—	—	—	—	1	Culture collection	149/51
141 : · · ·	—	—	Phi, (K12)	—	+	—	1	Culture collection	RVC 2907
141 : 85B : 4	—	—	Phi, (17), (K12)	—	+	—	1	Culture collection	RVC 2907
141 : 85B, 88L : 4	—	—	(Phi), (17), (K12)	17	±	—	1	Culture collection	E 68
142 : · · ·	—	—	—	—	—	—	1	Culture collection	C 771
142 : 86B : 6	—	—	—	Phi, 17	—	—	1	Culture collection	C 771
143 : · · ·	8, 9	A3	—	—	—	+	1	Culture collection	4608
144 : · · ·	2, 3, 8, 28	A1, A3, G1	(Phi), (17), (K12)	—	—	—	1	Culture collection	1624
145 : · · ·	4, 12	A1, A4	—	—	—	—	1	Culture collection	1385(3)
146 : · · ·	1, 7, 8, 9, 28	A1, A2, A3, G1	—	—	—	—	1	Culture collection	CDC 2950/54

Discussion

Identification and type division on the basis of the phage pattern facilitate aetiological diagnosis and offer help in epidemiological studies. Comparing for pathogenicity the O antigens of *E. coli* strains occurring in the faeces of healthy infants and of those suffering from enteritis, ØRSKOV [18] stated that it was not the O antigen, but certain serofermentative groups occurring in the patients which determined the pathogenicity. In a more recent work, ØRSKOV *et al.* [7] described an immunoelectrophoretic classification of the extracts of *E. coli* strains and found a correlation between the immunoelectrophoretic pattern and 3 groups of diseases (infantile diarrhoea, dysentery-like and extra-enteric infections).

Considering the data mentioned above and those found by NICOLLE *et al.* [19] and by us, it may be concluded that diseases and epidemics are caused by certain serofermentative phage types of *E. coli* strains. Since the pathogenicity of more and more serogroups of *E. coli* has been proven, phage typing would become rather difficult if different type-phage sets were required for the typing of each *E. coli* group.

The correlation between phage sensitivity and antigen was studied by KAUFFMANN and VAHLNE [20] in 1945. They found that the non-capsulate forms of strains belonging to group O9 are phage sensitive, while the capsulate ones are phage resistant and that strains of the same serotype exhibit different sensitivity to phages. The correlation between capsular antigen and phage resistance was studied also by TOFT [21]. Phages affecting only capsular or acapsular forms or both were distinguished on the basis of the phage resistance of strains L⁺ and L⁻ and A⁺ and A⁻ belonging to serogroup O9. More recently, BORISOV *et al.* [22] found a correlation between the colicin and phage receptors and the OB antigens of group O26 and O111 strains.

STIRM [23] isolated K phages affecting only the capsular forms of *E. coli*. STIRM *et al.* [24] demonstrated that the spike organelles played a role in the adsorption and penetration through the polysaccharide gel functioning as receptor.

Studying various R mutants of *E. coli* with R-specific phages, SCHMIDT [25] found a characteristic phage reaction pattern for each type of basal lipopolysaccharide.

Our own studies performed thus far indicate that though no obvious correlation has been found between the antigens O, K or H and the sensitivity to phages, the serogroup can be extrapolated from the phage pattern. No unequivocal correlation between phage sensitivity and antigens O, K and H could be established. Further studies on this problem are planned taking into consideration the results of ØRSKOV *et al.* [7] and JANN *et al.* [26] on the biochemical and immunoelectrophoretic properties of the antigens O and K.

Methods for the classification of the phage types have been developed, by several authors for various *E. coli* strains. Methods for the phage type classification of *E. coli* strains belonging to serogroups O111, O55 and O26 have been elaborated by NICOLLE *et al.* [19], EÖRSI *et al.* [11, 12, 15] and by MILCH and DEÁK [13]. NICOLLE *et al.* typed a high number of strains originating from various geographical areas and found the results representative also from the epidemiological point of view [27]. Their method has later been extended and simplified [28]. Using phages isolated from the faeces of infants suffering from enteritis, EÖRSI *et al.* [11, 12, 15] and MILCH and DEÁK [13] divided strains belonging to groups O111, O55 and O26 into 7, 5 and 4 phage types, respectively. ADJAROV [29] elaborated a method for the typing of strains O111 and recommended the use of type-phages for the identification of strains O111 and O55. ACKERMANN *et al.* [30] classified strains O127 on the basis of their phage sensitivity. KAYSER [31] classified the strains O114 according to phage and biotypes which appeared suitable for the solution of epidemiological problems. BERCOVICI *et al.* [32] typing with 57 phages, found no characteristic phage pattern for either of the serogroups O4, O6, O8, O18, O20 and O25 isolated from the faeces of infants suffering from enteritis. They developed a scheme for each serogroup and could distinguish a total of 136 different phage types. Strains O20 and O4 were the most heterogeneous with phage types 14 and 11, respectively.

Typing of *E. coli* strains causing urinary tract infection was the purpose of the work by BROWN and PARISI [33]. Using 8 phages isolated from sewage, they divided serologically unidentified *E. coli* strains of 7 different serogroups (O1, O4, O6, O7, O11, O25 and O75) into phage types and they could type 74 of the 90 strains originating from infections of the urinary tracts. Except offering a possibility for the intraserogroup subdivision of the phage types, the number of strains studied was too small to establish any rule from the results.

PARISI *et al.* [34] used the above phages — with the addition of some other ones — for the typing of *E. coli* strains originating from urinary infections and some other sources. Fifty and 34.4%, respectively, of the strains were successfully typed. They stated that due to the easy and rapid procedure, phage typing was a new and suitable means for studying the epidemiology of urinary *E. coli* infections.

A method for the typing of *E. coli* strains pathogenic for animals was developed by SMITH and CRABB [35]. The *E. coli* strains isolated from the faeces of calves were divided into 71 types using phages isolated from the same source. The strains isolated from sick calves were different in phage type from those obtained from healthy ones (no serotyping of these strains was performed).

Our present experiments show that phage pattern, phage group, colicinogenic and lysogenic property, and the PP reaction offer a suitable means for

a finer intraserogroup differentiation as found for strains of serogroups O4, O18 and O111. In other serogroups, it is mainly the colicinogenic and lysogenic property which can be used for a further intraserogroup differentiation (O59, O124). The same results were obtained by DEÁK [36] with the typing of serogroup O124.

Since in our method testing for lysogeny refers only to phages released spontaneously and the method had to be developed so that it should primarily serve a rapid orientation, the result for lysogeny is considered to be valid only in positive cases. In serogroups O4 and O8 phage pattern and colicinogeny, while in serogroup O18 phage pattern and lysogeny could have been used for an intraserogroup differentiation. In our more recent material colicinogeny is frequent among strains O18 and thus their differentiation is possible also on the basis of colicinogeny. For the other serogroups, no general conclusion could be drawn due to the small number of cases studied.

Haemolysis was not considered in the intraserogroup classification since it had proved an unstable property though the first results obtained after receiving the sample have been included in the Tables.

The phage sets and the supplementary methods presented are suitable also for calling attention to the identity of *E. coli* strains not identified or unidentifiable serologically. For practical purposes, the serological identification of strains selected and considered identical on the basis of their phage pattern, colicinogeny, etc., seems to be a method more simple than serotyping.

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PRODUCTION OF CYTOPHILIC ANTIBODIES IN RABBITS AND MICE AGAINST *ESCHERICHIA COLI*

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Summary. The production of cytophilic antibodies in rabbits and mice against a smooth and a rough strain of *Escherichia coli* has been studied. Cytophilic antibodies for various cells using several *E. coli* antigens were investigated. Rabbit spleen cells, lymph-node cells as well as peritoneal macrophages from both rabbit and mouse were effective in adsorbing the homologous cytophilic antibodies. Polymorphonuclear cells did not possess the suitable receptors for cytophilic antibodies against both the test strains of *E. coli*. The cytophilic antibodies in case of 3662, a smooth strain of *E. coli*, were directed against the heat-labile antigen, possibly the flagellar antigen and not against somatic or capsular antigens. Lilly, a rough strain of *E. coli*, failed to produce cytophilic antibodies in both mice and rabbits. Passive intravenous transfer of cytophilic antibodies in rabbit against strain 3662 was confined to peritoneal macrophages.

Various micro-organisms have been shown to adhere to lymphoid cells of animals immunized with homologous organisms [1, 2]. Particulate antigens such as sheep erythrocytes may adhere to mouse macrophages *in vitro*. This adherence results in the formation of rosettes which could either be due to the presence of cytophilic antibody on the macrophage surface or to antibody coating the particulate antigens and subsequent binding to the cell surface.

Cytophilic antibodies are globulin fractions of normal or immune serum capable of attaching themselves to certain cells in such a way that these cells become subsequently capable of adsorbing specific antigens [3, 4]. The immunological reactions involving cells of the body and the infectious agents mediated by serum factors are of immense significance. Several types of cells such as lymphocytes, macrophages, monocytes, granulocytes and mast cells participate in the immune response and in related inflammatory processes. Some of them possess specific receptors to recognize substances which are released, modified or produced after antigenic stimulus. Since there is a paucity of information regarding the production of cytophilic antibodies in Gram negative bacterial infections, an attempt has been made to study the production of cytophilic antibodies in rabbits and mice against *Escherichia coli*.

Materials and methods

1. *Bacteria*. The smooth strain 3662 and the rough strain Lilly of *E. coli* maintained in the Department of Veterinary Microbiology and Immunology at Ontario Veterinary College, University of Guelph, were used.

2. *Surface characteristics of bacterial strains*. The tests recommended by WILSON and MILES [5] were used to study the surface properties of the bacteria. Susceptibility of the test strains to the bactericidal action of normal fresh sheep or rabbit serum was studied by the technique described by MITTAL [6].

3. *Antigens*. (a) *O antigen*. A single colony of the 3662 strain of *E. coli* from a bovine blood agar plate was transferred to tryptic soy broth (TSB) which was incubated at 37°C for 10 to 12 hours. Roux flasks containing tryptic soy agar medium were inoculated with the TSB culture and incubated at 37°C for 24 to 36 hours. The bacteria were removed from these flasks and were washed twice in sterile isotonic saline. The dense suspension of *E. coli* was heated at 121°C for 2 hours to destroy the K antigen. It was cooled, washed twice in saline and suspended in five times volume of cold acetone. The acetone dried cells were stored at 4°C and used as somatic antigen. The dried powder was used to prepare the antigen suspension by adding 1 mg of the acetone dried bacteria per ml of saline and used to coat the sheep red blood cells.

(b) *R antigen*. R antigen of the Lilly strain of *E. coli* was prepared like O antigen except that the dense suspension of *E. coli* was heated at 100°C for 1 hour.

(c) *K antigen*. The method of INGRAM *et al.* [7] was applied for the preparation of capsular antigen of *E. coli*. The capsulated *E. coli* strain 3662 was grown on bovine blood agar plate and subcultured on MacConkey agar. Mucoid colonies were picked from the MacConkey medium and tested for the presence of capsular antigen by the slide agglutination test using rabbit O and OK antisera. A part of the colony positive for K antigen was cultured in TSB. The broth was incubated at 37°C for 10 to 12 hours, spread in Roux flasks and grown for 24 to 36 hours. The growth was removed, washed twice in formalinized saline and the packed cells were immediately extracted with cold acetone. These cells were stored at 4°C and used as K antigen for coating the sheep red blood cells.

4. *Preparation of antiserum*. Four healthy adult rabbits and ten healthy albino mice of either sex were injected each with 0.5 ml and 0.1 ml respectively of a live 18–20-hour-old broth culture of the 3662 strain of *E. coli* by the intraperitoneal route 3 times at 3 day intervals. Similarly, another group of four rabbits and ten mice was injected with an 18–20-hour-old broth culture of the Lilly strain of *E. coli*. The broth cultures of both strains were killed by heating at 100°C for 1 hour. The same schedule of vaccination with heat-killed organisms was followed for different groups of animals as in the case of live organisms. Six days after the last injection, the animals which had survived the *E. coli* infection were anaesthetized with ether. Rabbits were bled directly from the heart and mice were bled from the heart after opening the thoracic cavity. The serum collected was stored at -20°C till used.

5. *Spleen and lymph-node cell suspension*. Suspension of normal rabbit spleen cells was prepared as follows. A healthy rabbit was anaesthetized with ether. Its spleen was removed aseptically and cells were collected by teasing a piece of spleen on a wire-mesh over cold Eagle's medium culture fluid to give a concentration of 20% (one volume of packed cells and four volumes of medium). The cell suspension was always used within 15 minutes of its preparation. The mesenteric lymph-node cell suspension was prepared in the same manner.

6. *Preparation of cell monolayers*. (a) *Peritoneal macrophages*. A normal mouse or rabbit was killed under ether anaesthesia. The abdominal skin was moistened with 75% ethanol and four and ten ml of Hanks' solution was introduced into the peritoneal cavity. After gentle agitation the washings were withdrawn from the peritoneal cavity and placed into sterile tubes. Two to three drops of the cell suspension were placed in the cavities of the glass slides. Monolayers were formed after incubation in a moist chamber at 37°C for 1 hour.

(b) *Blood leucocytes*. Two to three drops of freshly drawn blood were allowed to clot in the cavities of glass slides. The clot was removed after incubation in a moist chamber at 37°C for 30 min. The monolayer formed consisted mainly of neutrophils.

(c) *Spleen cells*. The monolayer of spleen cell suspension was also prepared like those of peritoneal macrophages.

7. *Test for cytophilic antibodies*. The cytophilic antibodies for lymphocytes were tested in a tube by the centrifugation-suspension technique and those for macrophages and polymorphonuclear cells were tested by the cell-monolayer method [8, 9]. The monolayers of peritoneal and spleen macrophages and blood polymorphonuclear cells prepared on cavity slides were washed with phosphate buffer saline (PBS) of pH 7.2. 0.1 ml of test serum was allowed to react in a moist chamber with the monolayer at 37°C for 30 minutes. Controls consisted

of monolayers treated with PBS or normal homologous serum. After incubation, the slides were washed four times with PBS. 0.1 ml of a 4-hour-old live culture of the test strain was placed on the monolayer and incubated in a moist chamber at 37°C for 30 minutes. The cavities were washed 3 times in PBS and stained by Giemsa's method for observation under microscope. Cells showing a rosette with at least two bacterial cells were considered positive for cytophilic antibodies. The highest dilution of serum that caused rosette formation with at least 50% of the cells was taken as the end-point.

8. *Passive transfer experiment.* An adult healthy rabbit was injected with 5 ml of fresh rabbit anti-live 3662 *E. coli* serum, and another rabbit with rabbit anti-heat-killed 3662 *E. coli* serum. Twenty-four hours later, blood was collected for preparation of the monolayers of polymorphonuclear cells. The rabbits were anaesthetized with chloroform, killed, and peritoneal fluid, spleen and mesenteric lymph-nodes were collected. Tests for detecting the sensitization *in vivo* of these cells with antiserum was performed *in vitro* by the methods described earlier.

Results

The surface characteristics of the *E. coli* test strains are shown in Table I. Strain 3662 was smooth and capsulated. Strain Lilly was rough and lacking both K and O antigens. Rabbit antiserum against live 3662 was found to contain rabbit macrophage and lymphocyte cytophilic antibodies against the live homologous strain only. The heat-killed organisms adhered non-specifically to the macrophages, spleen and lymph-node cells and blood polymorphonuclear cells. Heat-killed 3662 strain failed to stimulate the production of cytophilic antibodies in rabbits (Table II). The Lilly strain did not stimulate cytophilic antibodies in rabbits. Polymorphonuclear cytophilic antibodies were also absent. A minimum of 100 cells was counted for bacterial adherence. The results presented in Tables II, III and IV are representatives of the various replications of the test.

Table I

Surface characteristics of E. coli strains

Property	Strains	
	3662	Lilly
1. Stable in 0.85% saline at 37°C for 24 hours	yes	no
2. Stable in 0.2% saline solution	yes	no
3. Stable in physiological saline solution when heated at 100°C for 30 minutes	yes	no
4. Stable when grown in tryptic soy broth at 37°C for 18 hours	yes	no
5. Agglutination in acriflavine solution	no	yes
6. Susceptible to the bactericidal action of normal fresh sheep or rabbit serum	resistant	extremely sensitive

The data of passive transfer experiments presented in Table II show that only peritoneal macrophages were sensitized by the cytophilic antibodies to *E. coli*. Lymph-node and spleen cells remained unaffected by passive immunization.

Table II

Rosettes formed with strains 3662 and Lilly of E. coli against various rabbit cells when treated in vitro with homologous antiserum

Rabbit antiserum against <i>E. coli</i>	Antigen (live <i>E. coli</i>)	Per cent rosettes formed			
		Peritoneal macrophages*	Lymph-node cells**	Spleen cells*	Blood polymorphs*
Live 3662	3662	91	95	75	0
	Lilly	0	0	0	0
Heat-killed 3662***	3662	0	0	0	0
	Lilly	0	0	0	0
Live Lilly	3662	0	0	0	0
	Lilly	0	0	0	0
Heat-killed Lilly***	3662	0	0	0	0
	Lilly	0	0	0	0

* Tested for cytophilic antibody by cell monolayer method

** Tested for cytophilic antibody by centrifugation-suspension technique

*** Heat-killed organisms adhered non-specifically to various cells without prior sensitization with antiserum

Table III

Rosettes formed with live E. coli strain 3662 against various cells from rabbit passively immunized with homologous immune serum*

Antiserum against <i>E. coli</i>	Per cent rosettes formed			
	Peritoneal macrophages	Spleen cells	Lymph-node cells	Blood polymorphs
Live 3662	95	0	0	0
Heat-killed 3662**	0	0	0	0

* Injected intravenously with 5 ml of homologous anti-3662 *E. coli* serum 24 hours before the test

** Heat-killed organisms adhered non-specifically to various cells without prior sensitization with antiserum

The results obtained with mouse peritoneal macrophages treated with mouse anti-*E. coli* serum and various antigens of *E. coli* are summarized in Table IV. Cytophilic antibodies were only detected in antiserum against live 3662 organisms. Antiserum against live 3662 *E. coli* failed to show the presence of cytophilic antibodies against homologous capsular or somatic antigens when coated on sheep red blood cells. Neither live Lilly nor their R antigen coated on sheep red blood cells formed rosettes with macrophages treated with anti-Lilly or anti-3662 *E. coli* serum.

Table IV

Effect of treatment of mouse peritoneal macrophages with homologous anti-E. coli serum on their activity against E. coli antigens in vitro

Mouse antiserum against <i>E. coli</i>	Antigen (<i>E. coli</i>)	Cytophilic antibody* per cent (rosettes formed)
Live 3662	Live 3662	90
	Sheep red blood cells coated with 'K' antigen of 3662	0
	Sheep red blood cells coated with 'O' antigen of 3662	0
	Live Lilly	0
	Sheep red blood cells coated with 'R' Antigen of Lilly	0
	Live 3662	0
Live Lilly	Sheep red blood cells coated with 'O' antigen of 3662	0
	Live Lilly	0
	Sheep red blood cells coated with 'R' antigen of Lilly	0

* Tested for cytophilic antibody by cell-monolayer method

Discussion

The results presented in Tables II and III indicate that cytophilic antibodies in rabbits are produced only against the live smooth strain of *E. coli*. Rabbit cytophilic antibodies against strain 3662 reacted equally well with rabbit peritoneal cells, spleen cells and lymphocytes. Cytophilic antibodies against 3662 were, however, not demonstrable for blood polymorphonuclear cells. The Lilly strain probably did not possess the right antigens to provoke cytophilic antibodies.

When capsular or somatic antigens of strain 3662 coated on the sheep red blood cells were used as test antigens instead of live organisms, cytophilic antibodies were not demonstrable in mouse anti-3662 serum. The results in Table IV show that cytophilic antibodies in mice are produced in response to the antigens possessed only by the live smooth organisms. It is suggested that this antigen could possibly be the flagellar antigen rather than capsular or somatic antigens.

Cytophilic antibodies in mice have been studied by several workers [10-13]. Mouse cytophilic antibodies to somatic antigens of *E. coli* were reported by PARISH [14] and were presumed by him to be 19S. UHR [15] studied the cytophilic antibodies for macrophages in guinea-pigs injected with *Salmonella paratyphi* flagellae with and without adjuvant. He used the whole live bacteria as the antigen for detecting cytophilic antibodies. The sera from guinea-pigs immunized with flagellae in adjuvant contained cytophilic antibodies detectable in this way but other sera did not. In the present investigation, both rabbits and mice were able to produce cytophilic antibodies to *E. coli* without using adjuvant when tested with whole live organisms as anti-

gen. PARISH [14] reported that red blood cells coated with somatic polysaccharide of *E. coli* adhered well to the cytophilic antibody-treated mouse macrophages. The present results indicate, however, that red blood cells coated with capsular or somatic antigen of strain 3662 failed to adhere to the macrophages pre-sensitized with antiserum.

ROWLEY *et al.* [16] reported evidence suggestive of the existence of mouse cytophilic antibodies to *Salmonella typhi-murium*. This was based on the ability of peritoneal cells from immune mice to protect normal mice against a challenge infection with the homologous organisms.

The data of Table III indicate that the cells of spleen or lymph-node tissues as well as blood polymorphonuclear cells cannot be sensitized passively by circulating antibodies as with peritoneal macrophages.

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PHENOTYPIC DEREPRESSION OF ENZYMES OF THE ISOLEUCINE-VALINE PATHWAY BY ISOLEUCINE IN PSEUDOMONAS AERUGINOSA

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Summary. The phenotypic derepressive effect by isoleucine in certain variants of *Pseudomonas aeruginosa* was followed by different means. *P. aeruginosa* strain 132 can be derepressed through the whole period of growth, while in the case of strains PAO1 and PAT2 derepression was detectable only during that phase of growth in which there was a spontaneous increase of those enzyme levels which participate in the biosynthesis of isoleucine and valine. A similar behaviour to that observed in wild-type strains was demonstrable in the case of methionine-requiring and uracil-requiring mutants in the presence of maximum and limiting concentrations of the particular growth requirement. The behaviour of leucine requiring mutants of strain PAO1 was the same as that of the wild-type in the presence of a leucine concentration allowing maximum growth. Derepression of enzymes of the isoleucine-valine pathway occurs by addition of isoleucine under conditions when growth is limited by leucine. It was found that the level of threonine deaminase activity was not only independent of experimental conditions, but was not related to levels of the other three enzymes. However, the levels of transaminase B showed similar changes to those of acetohydroxy acid synthetase and dihydroxy acid dehydrase.

The phenotypic derepression of enzymes participating in the biosynthesis of isoleucine and valine has been studied in different microorganisms and has been shown to result from the effects of a variety of substances. In *Streptomyces rimosus* it results from α -ketobutyric acid and its precursors [1]; in *Mycobacterium pellegrino* valine is effective [2]; and in *Pseudomonas aeruginosa* isoleucine and its precursors caused phenotypic derepression [3]. The derepression in the case of *M. pellegrino* and *S. rimosus* can be attributed to the regulatory mechanism of the first common enzyme, acetohydroxy acid synthetase (AAS) [4, 5]; while in the case of *P. aeruginosa* the mechanism of derepression could not be interpreted by the properties of this enzyme. In this paper we describe experiments related to the derepressive effect of isoleucine in mutants of *P. aeruginosa*.

Materials and methods

The strains used throughout the experiments are collected in Table I. Genetic data of strains PAO1 and PAT2 are described by HOLLOWAY [6, 8].

The minimal medium and the experimental conditions were the same as described previously [3].

The procedures for determining enzyme activities were those previously reported [9] in which a crude extract was obtained by ultrasonication of the cells and followed by gel-filtration using Sephadex G 25 in the presence of cofactors. Enzyme activities were estimated according to standard methods; AAS activity was determined by the method of HALPERN and UMBARGER [10]; threonine deaminase according to SZENTIRMAI and HORVÁTH [11]; dihydroxy acid dehydrase by the method of WIXOM *et al.* [12] and transaminase B according to STRASSMAN *et al.* [13].

Specific activity was expressed as micromoles of end-product per milligram of protein per hour.

Table I
Strains used in the experiments

Strain	Description
<i>P. aeruginosa</i> 132	Wild-type strain
<i>P. aeruginosa</i> PAO1	Wild-type strain, formerly numbered 1C (ATCC 15 692)
<i>P. aeruginosa</i> PAO38	Leucine-requiring mutant, derived from parent strain PAO1 (ATCC 15 692)
<i>P. aeruginosa</i> PAT2	Wild-type strain, formerly numbered 2 (ATCC 15 693)
<i>P. aeruginosa</i> PAT301	Methionine-requiring mutant, derived from parent strain PAT2 (ATCC 15 693)
<i>P. aeruginosa</i> PAT635	Uracil-requiring mutant, derived from parent strain PAT2 (ATCC 15 693)

Results

Behaviour of wild-type strains. In accordance with the findings of CHIBATA *et al.* [14], we demonstrated isoleucine production by *P. aeruginosa* strain 132. Furthermore, a coordinate increase in the level of enzymes of the isoleucine-valine pathway, due to the effect of isoleucine and isoleucine precursors was observed. In the first series of experiments we mainly used toluene-treated cells. Subsequently, these experiments were repeated with cell-free extracts. The results obtained were compared with those found with *P. aeruginosa* strains PAO1 and PAT2 which do not excrete isoleucine. In the course of our experiments AAS activity was measured relative to growth. The results are indicated in Figs 1a and 1b.

It can be seen that AAS level of strain 132 which is suitable for isoleucine production was higher during the stationary phase of growth in comparison with the other two strains. A transitory increase in the level of enzymes of the isoleucine-valine pathway was demonstrable similarly to that first observed in *Escherichia coli* by UMBARGER and BROWN [15] and which has been observed in the case of all microorganisms [11] in which this pathway has been studied. Isoleucine exhibits a strong effect on this temporary increased enzyme synthesis and the derepressive state prevails throughout the whole period of

growth. In the case of the PAO1 strain, the initial enzyme levels were considerably lower and the rate of temporary spontaneous increase was also lower but the addition of isoleucine resulted in an increase which was not as pronounced as that found in strain 132.

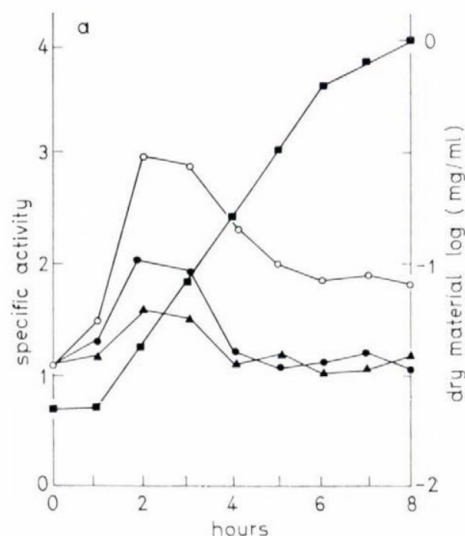


Fig. 1a. Effect of isoleucine and valine on AAS levels of *P. aeruginosa* strain 132. —●— no addition; —○— 300 µg/ml isoleucine; —▲— 200 µg/ml valine; —■— dry weight of cells with and without addition

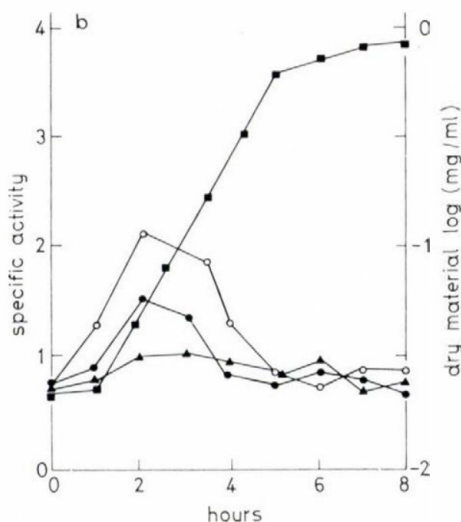


Fig. 1b. Effect of isoleucine and valine on AAS levels of *P. aeruginosa* strain PAO1 and PAT2. —●— no addition; —○— 300 µg/ml isoleucine; —▲— 200 µg/ml valine; —■— dry weight of cells with and without addition

Behaviour of auxotrophic mutants. The levels of enzymes of the isoleucine-valine pathway were followed in methionine-requiring and uracil-requiring mutants derived from the parent prototrophic strain PAT2. In the presence of growth factor concentrations giving maximum growth, auxotrophic mutants exhibited about the same enzyme activity as the parent strain and isoleucine effect was restricted only for the period of spontaneously increased enzyme level. Data for PAT301 — one of the auxotrophic mutants — are given in Fig. 2.

The behaviour of the leucine-requiring mutant PAO38 was essentially the same as that of the wild-type PAO1 when 100 $\mu\text{g/ml}$ leucine eliciting maximum growth was added to the medium (Fig. 3). Derepression was further increased on the addition of leucine at very low concentrations (20 $\mu\text{g/ml}$) and under various experimental conditions up to an eightfold increase in enzyme levels was observed. The most pronounced effect was attained with 300 $\mu\text{g/ml}$ isoleucine but at this concentration some inhibition of growth was observed. BAUERLE *et al.* [16], using *Salmonella typhi-murium*, found a considerable derepression of isoleucine-valine biosynthetic enzymes when leucine was used at a growth limiting concentration (20 $\mu\text{g/ml}$).

The coordinate character of isoleucine-valine enzymes. Using the data from the above experiments to select a particular time in the growth sequence for the measurement of enzyme activity, changes in the levels of enzymes participating in the biosynthesis of isoleucine and valine were followed with both wild-type and mutant strains. The results are summarized in Table II.

Table II

Coordinative character of the enzymes of the isoleucine-valine pathway

Strain	Additions $\mu\text{g/ml}$	Enzyme specific activity (micromoles of end-product per mg protein per hour)							
		Acetohydroxy acid synthetase		Threonine deaminase		Dihydroxy acid dehydrase		Transaminase B	
		A	B	A	B	A	B	A	B
132	none	2.0	1.1	1.8	1.9	0.40	0.38	0.36	0.34
	300 isoleucine	3.2	2.1	1.7	1.9	0.38	0.40	0.36	0.32
PAO1	none	1.6	0.8	2.1	1.8	0.31	0.28	0.30	0.26
	300 isoleucine	2.2	0.4	1.9	2.0	0.28	0.24	0.30	0.30
	100 leucine	1.4	0.6	1.9	1.8	0.28	0.30	0.26	0.30
PAO138	100 leucine + 300 isoleucine	1.9	0.7	1.7	2.0	0.25	0.24	0.26	0.28
	20 leucine	3.5	2.6	1.9	1.7	0.72	0.69	0.84	0.60
PAO38	20 leucine + 100 isoleucine	6.4	4.5	1.9	2.0	0.70	0.81	0.60	0.68

The specific activity of enzymes was assayed at 3 (A) and at 8 (B) hr

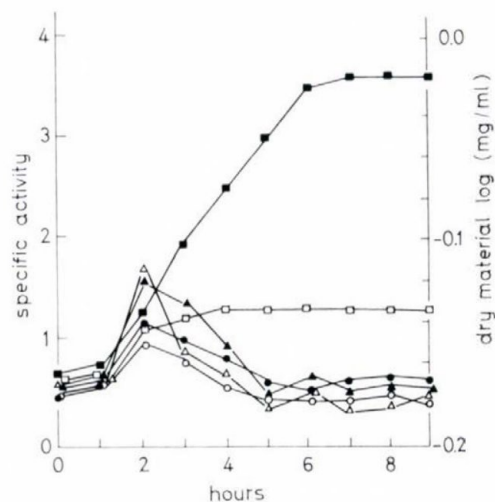


Fig. 2. Effect of isoleucine on AAS levels of *P. aeruginosa* strain PAT301. —○— in the presence of 20 $\mu\text{g/ml}$ methionine; —△— in the presence of 20 $\mu\text{g/ml}$ methionine + 300 $\mu\text{g/ml}$ isoleucine; —●— in the presence of 100 $\mu\text{g/ml}$ methionine; —▲— in the presence of 100 $\mu\text{g/ml}$ methionine + 300 $\mu\text{g/ml}$ isoleucine; —□— dry weight of cells in the presence of 20 $\mu\text{g/ml}$ methionine, —■— and in the presence of 100 $\mu\text{g/ml}$ methionine.

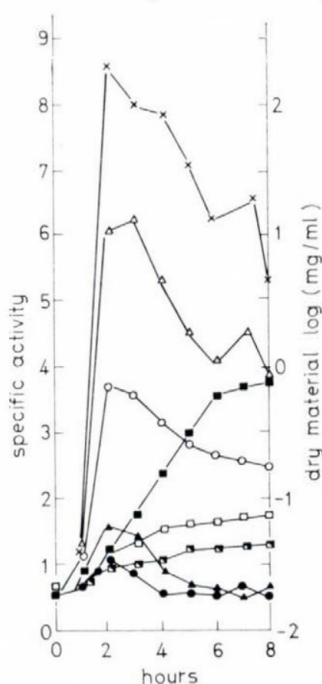


Fig. 3. Effect of isoleucine on AAS levels of *P. aeruginosa* strain PAO38. —○— in the presence of 20 $\mu\text{g/ml}$ leucine; —△— in the presence of 20 $\mu\text{g/ml}$ leucine + 100 $\mu\text{g/ml}$ isoleucine; —×— in the presence of 20 $\mu\text{g/ml}$ leucine + 300 $\mu\text{g/ml}$ isoleucine; —●— in the presence of 100 $\mu\text{g/ml}$ leucine; —▲— in the presence of 100 $\mu\text{g/ml}$ leucine + 300 $\mu\text{g/ml}$ isoleucine; —□— dry weight of cells in the presence of 20 $\mu\text{g/ml}$ leucine; —■— in the presence of 100 $\mu\text{g/ml}$ leucine; —■— in the presence of 20 $\mu\text{g/ml}$ leucine + 300 $\mu\text{g/ml}$ isoleucine, respectively.

It is seen that the level of threonine deaminase is not altered under the various conditions adopted. Furthermore, it can be concluded that threonine deaminase is not coordinately derepressed with any of the other three enzymes. However, it appears likely that an increase in the level of transaminase B is accompanied by an increase in the level of AAS. Likewise, an increase in dihydroxy acid dehydrase level is accompanied by a similar degree of change in the level of AAS.

Discussion

It is known that in the biosynthesis of isoleucine and valine several common enzymes are involved. As might have been expected, the unilateral loading of the regulatory system causes changes which can be interpreted only by considering the common biosynthetic pathway. Acetohydroxy acid synthetase plays a decisive role in the regulatory system and in most microorganisms it is inhibited by valine, which apparently affects the biosynthesis of the three-branched-chain amino acids. Valine inhibited growth only in the case of strain *E. coli* K12 [17], when the medium contained glucose. Earlier experiments with *P. aeruginosa* demonstrated that the levels of AAS are affected not only by valine — one of the end-products of the common pathway — but also when pyruvate acts as a substrate. It may be assumed that owing to the dissociation of the enzyme complexes, the high pyruvate concentration enhances the formation of a valine insensitive form of the enzyme, which form is suitable for the production of the required amount of isoleucine [18, 19].

The substrate affinity of the first common enzyme also exhibited a regulatory role in the tested microorganisms. The formation of the "active acetal" is competitively inhibited by the other substrate, α -ketobutyric acid. In the second step, the affinity of enzymes for α -ketobutyric acid becomes more significant, and accordingly, formation of isoleucine is ensured even at a very low concentration of α -ketobutyric acid.

In the case of *P. aeruginosa* strain 132, a derepressed state was observed which was suitable for isoleucine production in the presence of isoleucine and isoleucine precursors. It is not yet possible to explain the results on the basis of our current knowledge of the properties of AAS.

Our experimental results differ from those obtained by MARINUS and LOUTIN [20, 21]; the difference could be explained by the different behaviour of the strain used. In contrast to their results we obtained low threonine deaminase activities with *P. aeruginosa* strain 132 and its mutants, regardless of experimental conditions. In certain experiments, e.g. with a leucine-requiring strain, the activity of transaminase B and dihydroxy acid dehydrase changed parallel with the activity of AAS when various leucine concentrations were employed, indicating the coordinative synthesis of the enzyme in ques-

tion. Further experiments did not, however, support this hypothesis because with the leucine requiring strain, derepression stimulated by the effect of isoleucine caused an increase in the level of AAS which was not followed by any change in the levels of dihydroxy acid dehydrase and transaminase B. Most probably, from the enzymes participating in the isoleucine-valine biosynthesis, the regulation of AAS synthesis is functioning independently from the regulation of transaminase B and dihydroxy acid dehydrase biosynthesis. The formation of threonine deaminase is independent of both groups of enzymes. The specific activity of threonine deaminase was found to be exceedingly low depending on cultural conditions and genetic changes. Our results, together with those of MARINUS and LOUTIT, clearly indicate the difference between *P. aeruginosa* and *E. coli* with respect to both enzyme regulation and the arrangement of genes on the chromosome [22–24].

Experiments with various auxotrophic mutants indicated that, in the case of wild-type strain PAO1, which is unsuitable for isoleucine production, the derepressive effect of isoleucine has a transitory character, prevailing only during the spontaneously increasing period of growth. The behaviour of the methionine-requiring mutant was the same as those obtained with the wild-type strain PAO1. However, a different behaviour was observed with a leucine-requiring strain, which may serve to explain the derepressive effect of isoleucine.

As was demonstrated for the concentration of leucine providing maximum growth, the behaviour of mutants was the same as was observed for the wild-type strains. The derepression of enzyme of the isoleucine-valine pathway, as was expected occurred upon limiting the concentration of leucine, which may further be increased in the presence of small amounts of isoleucine. This finding presumably indicates that the continual derepressive effect of isoleucine is connected with the regulatory mechanism of leucine synthesis, and isoleucine functions through this pathway.

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SEROLOGICAL RELATIONSHIP BETWEEN PSEUDOMONAS AERUGINOSA AND ENTEROBACTERIACEAE

I. RELATIONSHIP OF PSEUDOMONAS AERUGINOSA O ANTIGENS TO SALMONELLA, ARIZONA AND CITROBACTER*

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Summary. By agglutination test with cultures heated at 100°C for 2½ hours, then at 130°C for 1 hour, 23 *Pseudomonas aeruginosa* strains representing all serological groups and subgroups of LÁNYI's antigenic schema have been examined for relationship to 49 *Salmonella*, 37 *Arizona* and 48 *Citrobacter* O antigen type strains. In view of a fairly frequent incidence of aspecific agglutinations, only reactions at titres 1 : 160 or above in at least three different batches of the serum were regarded as representing a relationship.

A major bilateral relationship of the a,b—a,c variety has been revealed between *Ps. aeruginosa* O7a,7c and *Arizona* O19. More important unilateral reactions were (sera/antigens): *Salmonella* O21/*Ps. aeruginosa* O3d,3f, O8, O9; *Salmonella* O45 — *Arizona* O11/*Ps. aeruginosa* O4a,4b, O4a,4c, O4a,4d; *Salmonella* O61 — *Arizona* O26/*Ps. aeruginosa* O13; *Arizona* O24/*Ps. aeruginosa* O7a,7b, O7a,7c; *Arizona* O29/*Ps. aeruginosa* O11; *Citrobacter* O6,4b,5b/*Ps. aeruginosa* O9; *Citrobacter* O8a,8b/*Ps. aeruginosa* O4a,4b, O4a,4c, O4a,4d; *Citrobacter* O16/*Ps. aeruginosa* O11; *Citrobacter* O22/*Ps. aeruginosa* O11; *Citrobacter* O39/*Ps. aeruginosa* O13; *Ps. aeruginosa* O3c/*Arizona* O8; *Ps. aeruginosa* O3a,3d/*Arizona* O8, *Citrobacter* O32, *Citrobacter* O35.

In recent years several attempts have been made at showing serological relationships between bacteria belonging to different taxonomic units. Investigations into this problem are partly of theoretical interest, as the results may be useful in studying systematic connections of bacteria. On the other hand, the knowledge of antigenic relationships is of importance in diagnostic work, especially in view of the immunofluorescent tracing of pathogenic organisms.

This study was undertaken in view of the fact that, although in recent years *Pseudomonas aeruginosa* antigens have been characterized by several authors, no systematic studies have been performed on the antigenic relationship of this organism to *Enterobacteriaceae*.

Materials and methods

Bacterial strains maintained in our collection were used. A total of 49 *Salmonella* type strains represented all O antigens of the Kauffmann—White schema from group O1, 2, 12 to group O61 and antigen Vi. Most of these cultures were of Kauffmann's series of type strains.

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Examination of *Arizona* strains was performed on the basis of the Edwards—Ewing schema [1]; the 37 type strains representing all antigens from O1 to O32 were received from the National Collection of Type Cultures, London. A total of 48 *Citrobacter* type strains obtained from the Culture Collection, Institute of Epidemiology and Microbiology, Prague, were employed for the determination of O groups 1—32 [10] and of provisional O groups 33—42 [9]. The 23 *Ps. aeruginosa* type strains represented all O groups and subgroups of LÁNYI's schema [6, 7].

Preparation of immune sera. *Salmonella*, *Arizona* and *Citrobacter* sera were prepared in rabbits by the usual technique with antigens heated at 100°C for 2½ hours. *Salmonella* sera O1, 2, 12, O4, 5, 12 and O9, 12 were prepared with formalinized cultures of non-flagellate strains, serum Vi with a living culture of *S. typhi* strain 965. *Ps. aeruginosa* antigens were heated at 75°C for 1 hour [6]. Sera showing homologous titres in the range 1 : 1280—1 : 10240 were used.

Tube agglutination. All agglutinations (except those with antigen Vi) were performed with bacterial suspensions heated first at 100°C for 2½ hours in saline, then at 130°C for 1 hour in glycerol [6]. For testing *Salmonella* and *Arizona* antigens, suspensions heated at 100°C for 1 hour were also used.

Tube agglutination was carried out in 0.5 ml serum dilutions with 0.05 ml bacterial suspension which had been adjusted to a standard density giving 74% transmittance in 1 : 10 dilution in 1 cm cuvettes of the Beckman DU spectrophotometer set at 0.03 mm slit and 530 mμ wavelength. Screenings were performed in 5 tubes for each individual serum at dilutions ranging from 1 : 20 to 1 : 320. The tubes were read after incubation in water bath at 50°C for 20 hours (for Vi antigen at 37°C for 20 hours).

Absorption of agglutinins was carried out by the usual technique. For details, see reference [6].

Results

Comparison of methods for testing antigenic relationships. In the beginning of the experiments tube agglutinations in *Ps. aeruginosa* O sera were performed with *Salmonella* and *Arizona* suspensions heated at 100°C for 1 hour. Comparison of these antigens and bacteria heated first at 100°C then at 130°C showed that the latter were more sensitive for the demonstration of antigenic relationships. After exposure to 130°C the antigens gave more easily readable reactions and the titres usually increased by one or two grades. Suspensions prepared in this manner were at least as stable as those heated at 100°C provided that S forms had been selected. The method was useful at the same time for the detection of cultures tending to undergo R variation.

In view of this finding, agglutination tests with *Salmonella* and *Arizona* antigens were repeated by using antigens heated at 130°C. *Citrobacter* cultures were examined only by this technique. For testing *Ps. aeruginosa* suspensions in *Enterobacteriaceae* sera, only bacteria treated in this manner were used throughout the experiments, since simple boiling of *Ps. aeruginosa* failed to give a sufficient antigen [6].

Aspecific agglutinations. In view of a fairly great number of aspecific reactions appearing in polyvalent sera, screening tests for antigenic relationships were performed in individual sera each representing one serological group or subgroup. Even in this manner agglutinations not associated with specific antibodies appeared frequently. Table I gives an account of the incidence of interspecies reactions obtained at first examination in unselected individual sera.

Table I

Agglutinations in unselected sera between Salmonella, Arizona, Citrobacter and Ps. aeruginosa

Sera		Antigens		Total No. of tests*	Distribution of tests according to titres					
Species	No.	Species	No.		negative (<1:20)	1:20	1:40	1:80	1:160	1:320 or above
<i>Salmonella</i>	49	<i>Ps. aeruginosa</i>	23	1127	689	190	147	67	27	7
<i>Arizona</i>	37	<i>Ps. aeruginosa</i>	23	851	665	101	63	10	6	6
<i>Citrobacter</i>	48	<i>Ps. aeruginosa</i>	23	1104	571	163	168	87	42	73
<i>Ps. aeruginosa</i>	23	<i>Salmonella</i>	49	1127	963	84	52	21	1	6
<i>Ps. aeruginosa</i>	23	<i>Arizona</i>	37	851	641	93	91	19	3	4
<i>Ps. aeruginosa</i>	23	<i>Citrobacter</i>	48	1104	884	112	61	31	3	13

* Screening tests for antigenic relationships were performed in serial dilutions of individual sera ranging from 1:20 to 1:320 with bacterial suspensions heated at 100°C for 2½ hours, then at 130°C for 1 hour. Homologous titres of the sera were 1:1280—1:10240

The overwhelming majority of reactions recorded at screenings failed to appear in other batches of sera prepared with the same strain in different rabbits. Certain sera tended to agglutinate at low titres nearly all the antigens tested. The incidence of aspecific reactions was associated with individual rabbits and was independent of the serogroup of the strain used for immunization and of the homologous titre of the serum. *Enterobacteriaceae* sera + *Ps. aeruginosa* antigens reacted somewhat less frequently than *Ps. aeruginosa* sera + *Enterobacteriaceae* antigens; the total number of negative tests (titres lower than 1:20) was 1925 (62.4%) in the former and 2488 (80.7%) in the latter group of screening tests. Titres of 1:160 or above were considered suspicious of indicating major antigenic relationships and the sera and antigens concerned were subjected to further examination.

Antigenic relationships. Positive agglutinations obtained at the screening tests were regarded as representing true antigenic relationships only after confirming the results in at least three batches of the serum originating from different stocks of rabbits immunized with different preparations of the antigen. These examinations showed that 162 out of 191 1:160 or higher titre agglutinations were aspecific reactions. Accordingly, only 29 agglutinations represented really existing major antigenic relationships. From Tables II and III it is evident that, with a single exception, the relationships were unilateral. The agglutination titre of the related antigens was usually several grades lower than the homologous titre.

As close antigenic relationships have been described to exist between certain *Salmonella* and *Arizona* and even between these organisms and *Citrobacter* strains, agglutinations in sera for the related *Salmonella*-*Arizona*-

Table II

Specific reactions of Ps. aeruginosa antigens in Salmonella, Arizona and Citrobacter sera

Serum*	Homologous titre**	Titre with <i>Ps. aeruginosa</i> antigens**
<i>Salmonella</i> O21	5120—10240	O3d,3f 160—320; O8 160—320; O9 80—320
<i>Arizona</i> O22	1280	O3d,3f 80 ; O8 80 ; O9 80—320
<i>Salmonella</i> O45	5120—10240	O4a,4b 160—640; O4a,4c 160—320; O4a,4d 80—160
<i>Arizona</i> O11	2560—5120	O4a,4b 160 ; O4a,4c 80 ; O4a,4d 40—80
<i>Salmonella</i> O59	5120	O7a,7b 20 ; O7a,7c 0 ; O11 0
<i>Arizona</i> O19	2560—10240	O7a,7b 160—640; O7a,7c 320—1280; O11 320—640
<i>Salmonella</i> O60	5120	O7a,7b 0 ; O7a,7c 0
<i>Arizona</i> O24	2560—5120	O7a,7b 80—320; O7a,7c 80—320
<i>Salmonella</i> O61	2560—5120	O13 640—1280
<i>Arizona</i> O26	2560—5120	O13 1280
<i>Arizona</i> O29	1280—5120	O11 160—1280
<i>Citrobacter</i> O5a,5b,4b	2560—5120	O9 0
<i>Citrobacter</i> O6,4b,5b	10 240	O9 160
<i>Citrobacter</i> O8a,8b	5120	O4a,4b 1280; O4a,4c 160; O4a,4d 160
<i>Citrobacter</i> O8a,8c	5120	O4a,4b 40 ; O4a,4c 0 ; O4a,4d 20
<i>Citrobacter</i> O16	5120	O11 160
<i>Citrobacter</i> O22	2560—5120	O11 160
<i>Citrobacter</i> O39	5120—10240	O13 160—320

* Each antigen was tested in at least 3 different batches of serum

** The antigens were heated at 100°C for 2½ hours, then at 130°C for 1 hour

Citrobacter serogroups were compared. The results are presented in Tables II and III. The relationship of *Ps. aeruginosa* antigens to related or identical *Salmonella* and *Arizona* antigens was evident for pairs *Salmonella* O21–*Arizona* O22, *Salmonella* O45–*Arizona* O11, *Salmonella* O61–*Arizona* O26, but not for pairs *Salmonella* O59–*Arizona* O19, *Salmonella* O60–*Arizona* O24. *Salmonella* serum O4,5,12 failed to agglutinate *Ps. aeruginosa* O11, while *Citrobacter* serum O22, which gives a definite cross-reaction with *Salmonella* B group, reacted with *Ps. aeruginosa* O11.

A bilateral relationship was demonstrated only between *Arizona* O19 and *Ps. aeruginosa* O7a,7c. The result of cross-absorption experiments is

presented in Table IV. It is seen that the relationship was of the a,b—a,c variety and that neither the *Arizona* nor the *Ps. aeruginosa* culture decreased considerably the titre of each other's serum.

Table III

Specific reactions of Salmonella, Arizona and Citrobacter antigens in Ps. aeruginosa sera

Serum*	Homologous titre**	Titre with Enterobacteriaceae antigens**
<i>Ps. aeruginosa</i> O3c	1280—5120	<i>Arizona</i> O8 160—320
<i>Ps. aeruginosa</i> O3a,3d	2560—10240	<i>Arizona</i> O8 80—320, <i>Citrobacter</i> O32 160, <i>Citrobacter</i> O35 80—320
<i>Ps. aeruginosa</i> O7a,7c	1280—5120	<i>Arizona</i> O19 160—320, <i>Salmonella</i> O59 20—40

* Each antigen was tested in at least 3 different batches of serum

** The antigens were heated at 100°C for 2½ hours, then at 130°C for 1 hour

Strains used in the experiments (Tables II and III) were: *Salmonella* O21 99 and 10239 (OKI); O45 1061 and 798; O59 10210; O60 1523; O61 10250 (OKI); *Arizona* O8 CDAI 426; O11 142—56; O19 Pc 166; O22 So 116; O24 Pc 196; O26 261—58; O29 2907—58; *Citrobacter* O5a, 5b, 4b Be 54/57; O6, 4b, 5b Be 54/57; O8a, 8b Be 62/57; O8a, 8c Be 106/57; O16 Be 80/57; O22 Be 86/57; O32 Be 103/57; O35 H310a; O39 LG 1099; *Ps. aeruginosa* O3c 170004; O3a, 3d 170005; O3d, 3f 170007; O4a, 4b 170008; O4a, 4c 170009; O4a, 4d 170010; O7a,7b 170015; O7a, 7c 170016; O8 170017; O9 170018; O11 170021; O13 170023

Table IV

Analysis of the antigenic relationship between Arizona O19 and Ps. aeruginosa O7

Antigen	Serum						
	<i>Arizona</i> O19 unabsorbed	<i>Ps. aeruginosa</i> O7a, 7b unabsorbed	<i>Ps. aeruginosa</i> O7a, 7c unabsorbed	<i>Arizona</i> O19 absorbed by O7a, 7b	<i>Arizona</i> O19 absorbed by O7a, 7c	<i>Ps. aeruginosa</i> O7a, 7b absorbed by O19	<i>Ps. aeruginosa</i> O7a, 7c absorbed by O19
<i>Arizona</i> O19	10240	40	320	5120	2560	0	0
<i>Ps. aeruginosa</i> O7a,7b	320	2560	1280	0	0	1280	640
<i>Ps. aeruginosa</i> O7a,7c	1280	640	5120	40	0	320	1280

Discussion

Extensive investigations into the serology of *Enterobacteriaceae* have revealed numerous antigenic interrelationships between genera of the family. The findings were reviewed by KAUFFMANN [5], EWING *et al.* [2], SEDLÁK and RISCHE [8] and SEDLÁK and ŠLAJSOVÁ [9].

Systematic studies on antigenic relationships within *Pseudomonadaceae* and between *Pseudomonadaceae* and *Enterobacteriaceae* have not been published. Even incidental observations are very few in number. When performing routine examinations of infants' faecal specimens, WIEDERMANN and FLAMM [11] observed that bacteria picked off *Ps. aeruginosa* colonies frequently agglutinated in *E. coli* O26 : B6 serum. They showed that their isolates were antigenically related to, but not identical with, *E. coli* O26 : B6. HORCH [4], studying the serology of *Ps. aureofaciens*, showed that the 4 antigenic groups of the organism were not related serologically to the *Ps. aeruginosa* type strains of HABS [3].

The present studies revealed few antigenic relationships of *Ps. aeruginosa* to *Salmonella*, *Arizona* and *Citrobacter*. From a comparison of Table I with Tables II and III it is evident that interspecies agglutinations obtained at the screening examinations were mainly aspecific reactions. True serological relationships confirmed in different batches of the sera were, with a single exception, unilateral ones.

Some *Ps. aeruginosa* antigens were agglutinated by sera for *Salmonella*, *Arizona* or *Citrobacter* serogroups themselves interrelated with one another. In 3 out of 6 such cases the *Ps. aeruginosa* antigen reacted with both the *Enterobacteriaceae* sera concerned; this means that the related *Ps. aeruginosa* factor was part of the main antigen responsible for the intrafamilial relationship. In the remaining 3 *Ps. aeruginosa*—*Enterobacteriaceae* relationships the *Ps. aeruginosa* culture was agglutinated only by one of the corresponding *Enterobacteriaceae* sera, indicating that the two strains shared minor partial antigens absent from the other *Enterobacteriaceae* strain.

In view of the usually medium titre and unilateral agglutinations between *Ps. aeruginosa* and the *Enterobacteriaceae* groups examined, and also of the fact that the *Enterobacteriaceae* antigens involved in the interfamilial serological relationships occur infrequently in routine materials, it may be concluded that the really existing relationships are negligible as a source of error in diagnostic work. The unexpected frequent incidence of antibodies not associated with confirmed antigenic relationships should be considered more seriously in causing errors in procedures based solely on antigen—antibody reactions.

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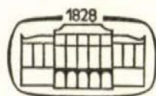
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PROCEEDINGS
OF THE INTERNATIONAL SCIENTIFIC MEETING
ON THE PROBLEMS OF LISTERIOSIS

HELD IN PÉCS, OCTOBER 6-7, 1972

ORGANIZED BY THE SOUTHERN HUNGARIAN BRANCH OF THE
HUNGARIAN ACADEMY OF SCIENCES AND BY THE
INSTITUTE OF MICROBIOLOGY, UNIVERSITY MEDICAL SCHOOL,
PÉCS, HUNGARY

OPENING SESSION

Welcoming Speech by Academician J. Tigyí, President of the Pécs Committee of the Hungarian Academy of Sciences

Dear Colleagues, Ladies and Gentlemen,

It is a very great honour and privilege to have the opportunity to greet this international conference on the problems of listeriosis. On behalf of the local Committee of the Hungarian Academy of Sciences I want to express our thanks to all the participants for their coming to Pécs and we are glad that the most prominent specialists of the listeriosis problem are present and therefore it is our firm belief that this meeting will be a significant step in furthering advances in this field.

The science policy of our Academy is in the favour of such small scientific meetings because we all have the experience that huge congresses are not able to discuss the real essence of the problems and there is an inverse ratio between size and efficiency of scientific meetings. Therefore we have supported the initiation of the Institute of Microbiology in organizing this conference. Under the direction of Professor RAUSS, Dr. RALOVICH was especially active and industrious in its realization.

Besides your scientific work I should like to suggest you to have a look at our town. Pécs is the oldest scientific centre and at the same time the most rapidly developing community in Hungary. The scientists of Pécs work hard now to create here one of the most significant centres of science in Hungary. We hope that our effort will result in building up here Hungary's Cambridge in a few years.

I should like to wish you successful scientific discussions and a good time in our town, and to enjoy all the facilities the organizers and the citizens of Pécs are offering you with friendship and hospitality.

With these sentences I declare open the International Scientific Meeting on the Problems of Listeriosis.

Welcoming Speech by Professor B. Boros, Rector of the University Medical School, Pécs

Ladies and Gentlemen, Distinguished Guests,

In the name of the University Medical School of Pécs, I acknowledge with pleasure the fact that we have repeatedly been honoured by conferences of various Scientific Societies. This time we greet a meeting organized by the

Southern Hungarian Branch of the Hungarian Academy of Sciences and by the Institute of Microbiology of our University, as a meeting which directs attention to an important question of public health. In the name of the Council of our University and of the teaching staff, let me greet warmly the foreign and Hungarian participants of this Conference.

The progress of scientific methods needs more and more cooperation between theoretical Institutes and Departments. When the policy of science attributes utmost importance to interdepartmental, complex research projects, then the organizers do indeed a good service, presenting an example for the importance and necessity of multisided research. The present program reveals some important findings regarding a disease which, though relatively rare, from the point of view of human and veterinary medicine, is significant. At the same time this meeting proves the outstanding role of preventive medicine in our socialist type public health.

We are glad to see that due to the scientific activities of the Institute of Microbiology, together with several departments, the knowledge of the bacteriology and epidemiology of listeriosis has considerably been enriched. This time we can learn also the opinions of our guests from abroad. I hope that the present Conference may give inspiration to further investigations.

When greeting the Conference once again, I wish a prosperous and useful work to all the participants.

**Opening Address by Professor K. Rauss,
Director of the Institute of Microbiology,
University Medical School, Pécs**

Ladies and Gentlemen,

It is a great pleasure for me to welcome you on the occasion of coming to our country and visiting our town Pécs to participate at this Conference. This meeting — I hope — is an important milestone in the investigation of listeriosis in Hungary. Therefore, please, allow me to give you a brief summary of the achievements we have done so far. A more detailed presentation of the results will be given in the lectures and discussions.

In Hungary the first occurrence of listeriosis was verified in sheep in 1952 by the veterinarians Mócsy and his team. Since that time, widespread research has been carried out, mainly in the Institutes of the University of Veterinary Science and the Central Veterinary Institute together with its branch offices as well as in some country laboratories, like the Laboratory of Meat Control Service in Pécs. On the basis of their results, these institutes and laboratories have a strong control over the problems of animal hygiene in our country and if it is necessary, they are able to intervene with effective

preventive measures. It is very fortunate that there is a good cooperation between veterinary and human doctors tackling this problem. As regards human listeriosis in Hungary, investigations were started rather late. This was connected with the fact that in the fifties much more serious infectious diseases had to be dealt with, e.g. typhoid fever, dysentery, poliomyelitis. Though the first warnings were hinted in 1956 by pathologists, ten years went by until RODLER and his coworkers have published the first bacteriologically verified human case of listeriosis. During that period, biological and other investigations were carried out in respect to listeriosis in the Institute of Microbiology, Semmelweis University Medical School of Budapest, and obstetricians have tried to clarify the significance of *Listeria monocytogenes* in their own field. For this purpose mainly serological reactions were used.

Here in our Institute, listeriosis investigations have been carried out since 1964. We reported our first results in Leipzig in 1968, when we suggested

- (1) a standardization of the methods;
- (2) examination of the aetiological role of *L. monocytogenes*;
- (3) continuation of the epidemiological survey.

In 1969, Dr. G. HABÁN, the deputy director of the National Institute of Public Health, organized a round-table conference for Hungarian physicians and veterinarians. As a result of this, an agreement has been reached concerning the diagnostic and methodologic questions of listeriosis. At the same time, possibilities were created to perform official examinations at every Public Health station. The use of a selective culture medium, developed in our institute, gave further impetus to the work.

I must mention the name of Dr. E. MÉRŐ from the National Institute of Public Health whose laboratory work as well as organizational and guiding activity had an exceptional importance.

Today's task is now to outline further goals on the basis of the papers given at this Conference. One of our main goals is to establish a fruitful international cooperation among the experts of different countries making our work more effective by a mutual exchange of opinions.

Finally, I wish a lively scientific atmosphere to the Conference. At the same time, I take the opportunity to express my thanks to Academician J. TIGYI, president of the Southern Hungarian Branch of the Hungarian Academy of Sciences, who has — in many ways — made this meeting possible. Thank you.

REVIEWS

NEW OUTLOOK ON THE EPIDEMIOLOGY AND EPIZOOLOGY OF LISTERIOSIS

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Since its discovery in the early twenties by MURRAY, WEBB and SWANN [*] in epizootics of rabbits and guinea pigs listeriosis has been considered a zoonosis. This view was supported by subsequent findings of PIRIE (1927) [*] in South Africa during plague studies in the course of which *Listerella* — later on designated *Listeria* by PIRIE (1940) [*] — was identified as cause of an epizootic in gerbilles (*Tatera lobengulae*) and the disease caused by small, non-sporulating gram-positive rods was named Tiger River Disease in those days.

Shortly thereafter listeriosis was discovered by GILL (1931, 1933) [*], as cause of circling disease in sheep in New Zealand and a gad fly *Oestrus ovis* was thought to be the transmitting agent. Enzootic and epizootic occurrences of the disease among domestic and wild animals were subsequently reported from the U.S.A., Australia, England, Germany and many other places. In contrast to the increasing frequency of findings in more than 50 species of the animal kingdom which included many sporadic cases, listeriosis remained rare in man. Up to 1950 not more than 70 sporadic cases had been identified, this figure having now risen to approximately 5000 all over the world.

The basic concept that listeriosis is primarily a zoonosis is now changing among the listeria-minded research workers whose number has always been and continues to be rather small.

As early as 1935 SEASTONE [*] had attempted to relate serological differences between a number of *Listeria* strains collected from different areas to their host. He thereby discovered 2 serologically distinct types of which the American animal strains had been isolated from ruminants.

Since the European strains belonged to serotype 1 of PATERSON's classification which was established some years later, and the American strains from ruminants represented the serotype 4, SEASTONE [*] suggested 2 sero-ecological groups: a "rodent" and a "ruminant" group. He thus followed an approach similar to the ecology of salmonellosis whereby certain *Salmonella* species or serotypes are more or less closely linked to specific hosts. For instance,

Salmonella typhi as well as *S. paratyphi-A* and *B* occur exclusively in man whilst *S. abortus-ovis* and *S. gallinarum-pullorum* have their infectious cycles among sheep and poultry, respectively. It is well known that such classical infectious cycles with a close host-parasite relationship are more an exception among the large crowd of *Salmonella* types, but this was not fully realized in the thirties.

In analogy to the differentiation of *Pasteurella multocida* into various species and subspecies, which were sometimes named according to the primary host, attempts were made to separate among *Listeria* a "horse type", a "cattle type", a "sheep type", etc. Soviet workers have even suggested a close linkage of pathogenicity to certain animal species and have claimed to transfer such restricted pathogenicity to other animal species by a process of adaptation of suitable cultures to certain animal species. This has, however, not been confirmed to our knowledge.

Table I
Serotypes of *L. monocytogenes*

Designation	O antigens	H antigens
PATERSON	SEELIGER— DONKER- VOET	
1	1/2a	I, II, (III) AB
	1/2b	I, II, (III) ABC
2	1/2c	I, II, (III) BD
3	3a	II, (III), IV AB
	3b	II, (III), IV ABC
	3c	II, (III), IV BD
	4ab	(III), V, VI, VII, IX ABC
4	4a	(III), (V), VII, IX ABC
	4b	(III), V, VI ABC
	4c	(III), V, VII ABC
	4d	(III), (V), VI, VIII ABC
	4e	(III), V, VI, (VIII), (IX) ABC
	4f	(III), V, XV ABC
	5	(III), (V), VI, VIII, X ABC
	6	(III), VII, VIII, XI ABC
	7	(III), XII, XIII ABC

On the basis of an adequate number of *Listeria* strains and by agglutination-absorption studies PATERSON proposed in 1938 [*] an antigenic schema which has become the valid basis of *Listeria* classification. *Listeria* was subdivided according to O and H antigenic factors into 4 distinct serotypes — see Table I. The study of isolates from different countries and hosts proved conclusively that the previous distinction of serological groups related to rodents and ruminants, respectively, could no longer be maintained. Both groups included strains isolated from human sources and from poultry. The only exception was at that time a new serotype “3” consisting of several strains from NYFELDT in Denmark. These had been isolated from human cases of suspected monocytosis during the early thirties. Although this serotype “3” has remained rare, it has been identified by us repeatedly among isolates from animals, too.

The obscure epidemiologic picture remained unchanged when listeriosis was observed with increased frequency in man. Some epidemiologic data may be discussed in the light of findings which were obtained in Germany between 1950 and 1968. In this period more than 2000 human cases were identified bacteriologically and a similar number by serological means [1].

Analyzing the findings in Germany a few conclusions are allowed which are in agreement with findings obtained in countries with comparable conditions of life and climate:

(i) With few exceptions the number of recognized and proved cases of human listeriosis increased from year to year.

(ii) The disease manifests itself primarily among new-born and individuals older than 45 years of age.

(iii) The clinical manifestations comprise mainly meningo-encephalitis, followed by septic infections and isolated organic involvement.

(iv) Only in foetal infection acquired during pregnancy *via* the trans-placental route, listeriosis takes the form of a septic granulomatous disease which either kills the foetus *in utero* or leads to abortion.

(v) As regards seasonality, the overwhelming majority of human cases is observed in late spring and throughout summer.

(vi) With few exceptions, the cases are sporadic in nature.

These exceptions relate to two epidemics which occurred during the years 1960 and 1961 in Bremen (Western Germany) [2] and a series of epidemics which started in 1949 in the district of Halle (Eastern Germany). There more than 180 clinical cases were confirmed bacteriologically in 1960, and another 160 human cases were identified in 1966 by ORTEL [3]. So far no conclusive evidence as to the mode of transmission and the primary reservoir of the agent was obtained. In the same area the largest incidence of cases had already been noted in the late forties by POTEL *et al.* [*].

If the incidence of human listeriosis is compared to its occurrence among animals, no evidence was detected on the German territories which would

support the view that the disease was likely to be transmitted to man from animals or animal products as is still suggested in several reports and textbooks. Of course, listeriosis has occasionally been transmitted from animals to man, for instance, an infection occurred in a veterinarian who experienced severe cutaneous and general listeriosis after having attended a cow with listeric abortion and who had not properly disinfected his arm.

Numerous purulent pustules appeared on the skin 2 days after the exposure and high fever developed. This patient could be saved by rapid proper diagnosis and antibiotic therapy (cf. KALKOFF and SCHIFF [*]). In a similar event a Dutch farmer succumbed to such an infection. An identical observation was made in a veterinarian in the United States.

Occasionally there was evidence that human listeriosis might have been acquired on the air-borne route by inhalation of dust from infected premises.

A direct transmission from animals has also to be considered in a few cases of laboratory infection. Two of them unfortunately occurred in our laboratory during animal experimentation although earlier experience (see later) indicated only little danger. Some cases of listeriosis have also been observed in animal keepers and occasionally among farmers who were in close contact with listeric animals and their excrements.

The common view that every case of human listeriosis among farmers, farmers' wives, animal keepers, butchers, etc., is exclusively or predominantly the result of professional exposure and therefore *per se* liable to social insurance or state compensation on the basis of recognized occupational disease can, however, not be accepted in such a generalization although it may be justified in sporadic cases. But these need to be ascertained for the legal proof.

This concept of listeriosis as not being a frequent occupational disease among possibly exposed individuals was supported by serological findings in our laboratory. Evidence of antibodies against *Listeria* was pronounced in individuals who were living together with pet animals such as dogs and cats in towns and villages, and also in persons who were in close contact with horses. These latter observations are recent and need further exploration. There was no serological evidence to support the view that listeriosis occurs preferably among the rural population, cattle breeders, etc. [4].

As a matter of fact, *Listeria monocytogenes* is a relatively mild pathogen for the healthy human adult. This was found quite unintentionally when we were conducting pathological studies with infected laboratory animals whereby direct contact of the unprotected hands with infected organs did not result in clinical illness or even serological evidence of subclinical infection. The two aforementioned cases occurred among two female workers who had handled large numbers of infected mice and had apparently become infected by exposure to highly virulent organisms.

The situation is, however, quite different during pregnancy and in early life. The clinical disease of the pregnant usually takes a very mild, often undetected course. This is in close agreement with similar observations in large animals [5].

Occasionally it was postulated that human infections are caused by the consumption of animal products contaminated with or infected by *Listeria*. Unpasteurized milk has been incriminated by NYFELDT in Denmark, WRAMBY in Norway and POTEL in East Germany during the first major outbreak of the disease in Halle (1949/50). KAMPELMACHER in the Netherlands has confirmed that during lactation a cow suffering from listeric mastitis shed large numbers of listeriae for several months. One such animal was kept by KAMPELMACHER quite unprotected. It excreted with its milk listeriae in great quantities into its environment, but no subsequent infection developed. The large outbreaks in Halle since 1966 cannot be milk-borne since — in the full awareness of such a possibility — production and sale of milk were rigidly controlled, and yet some hundreds of human cases developed.

Several European countries have extended bacteriological meat control to listeriosis; and meat from listeric animals is not to be used for human consumption. Yet such measures have neither resulted in the eradication of listeriosis in infected herds nor have they prevented human infections.

Another notable fact is the different seasonal distribution of cases and outbreaks of listeriosis in animals and man [6, 7]. The majority of cases in cattle occurs during the stabling period at the end of winter and in early spring. This is quite a uniform observation in Central and Western Europe and in regions of the U.S.A. with a similar climate [8, 9]. In the U.S.A., a certain periodicity with outbreaks of listeriosis recurring every 4 years has been noted. With the end of the stabling and with the feeding of fresh fodder, outbreaks of listeriosis tend to disappear rapidly and spontaneously.

Surprisingly, the main centre of outbreaks of listeriosis in Western and Eastern Germany with the highest number of human cases so far reported were in no way connected with a similar incidence of infections in animals. As a matter of fact, there has never been observed any parallel occurrence in these areas.

The lowering of resistance is obviously of great importance for the clinical course of infections in both animals and man. Listeriosis is being observed increasingly as secondary and may be a superimposed infection in patients suffering from leukaemia and neoplasms, and after haemodialysis in New Zealand [10], Belgium and Western Germany. Such patients may often have spent several months in the hospital without any contact with possibly infected animals. Moreover, the prolonged use of corticosteroids has been connected in a number of instances with the development of clinical listeric infections.

Notwithstanding the fact that, besides its occurrence in man, *L. monocytogenes* has been found to infect under natural conditions more than 50 animal species including birds, inmates of zoological gardens, rodents in desert climate and arctic regions, free-living animals and even trouts, the infectious agent lacks certain properties which makes it unlikely that one is dealing with a host-adapted pathogen. Strictly host-adapted pathogens usually cause in the specific host a very distinct disease and are either not transmissible to other species (for instance *S. typhi*, *Neisseria meningitidis*, *Shigella dysenteriae*) or may cause in other hosts a different clinical syndrome (e.g. salmonella gastro-enteritis, erysipeloid, Bang's disease, etc.). These differences are true not only for the clinical symptoms, but also for the localization, degree and kind of visceral involvement.

On this basis, typical zoonoses and anthroponoses have been separated from each other.

In listeriosis, however, a distinction on this basis is not feasible, for the clinical symptoms are astonishingly similar in animals and man. The same predilection for certain age groups, the analogous involvement of the central nervous system and of the meninges as well as the intra-uterine transmission of the disease in man as well as in carnivorous and herbivorous animals do not allow the differentiation of any classical "host specific" clinical syndrome. The histological structure of the tissue changes in human and animal listeriosis do not reveal any differences of importance. Listeriomas of the liver in a newborn baby and in an ovine foetus are indistinguishable by gross examination or histologically.

If infection of the brain occurs, the same foci are to be noted with vascular cuffing, necrosis and the preferred involvement of the same regions, such as the pons. Many other examples could be presented, e.g. the appearance of keratoconjunctivitis and the occasional occurrence of monocytosis in the circulating blood, which has very unfortunately led to the erroneous assumption that *Listeria* was once considered the cause of infectious mononucleosis.

In summary, the clinical syndromes of listeriosis show extreme conformity among animals and man. The course of the disease may be acute, subacute or chronic. Latent and asymptomatic infections might even represent the majority, whilst the acute cases have been considered as the "peak of an iceberg", the greater part of which is of unknown dimension. Such a view is supported by immunologic studies and by new bacteriological findings.

In spite of the established fact that in the epidemiology of listeriosis occasionally infectious chains lead from animals to man, the frequency of listeric antibodies in humans suggests that the prevalence of listeriosis in animals and man may be quite independent from each other. Exogenic factors

and environmental pollution with *Listeria* were considered to be perhaps the missing links to explain the lack of well established routes of infection.

The basis for this new concept was born when the epizootology of ovine listeriosis was successfully explored. Repeated epizootics during the stabling periods led to the examination of the silage fed. GRAY [11] succeeded in demonstrating *L. monocytogenes* in samples of poor silage, the organisms being occasionally observed in large quantities in the superficial layers and in poorly fermented sites which had not yet reached the low pH of good silage. Since acidity below pH 5.6 is lethal for listeriae, their survival could easily be explained in such little spots. These findings have repeatedly been confirmed in Iceland, Germany, the U.S.A. and elsewhere.

Thus the seasonal increase of ovine listeriosis was explained as a food-borne infection. The question, how listeriae contaminate silage, is still unsolved. The alkalinity of poor spots, the increased CO₂ concentration and the nutrient supply of silage all favour its growth. Listeriae will also grow at temperatures as low as +4°C, and the best flagellation is exhibited between 10 and 25°C and not at 37°C. The latter point may perhaps explain the poor development of H antibodies in humans and warm-blooded animals on the basis of poor flagella production.

SEELIGER and CHERRY [*] in 1956 have suggested that *L. monocytogenes* may be a soil-borne organism which shows many features of psychrophilic bacteria (see Table II).

Table II

*Features indicating that L. monocytogenes
may be a geophilic and psychrophilic bacterial species*

- (i) Growth at low temperatures (+3 to 4°C)
- (ii) Withstands sodium chloride and alkali at high concentrations
- (iii) High resistance to desiccation
- (iv) Best flagellation and motility at temperatures not higher than 25°C
- (v) Increasing frequency of isolation from dirt, soil, decaying vegetables
- (vi) Failure to produce host-specific clinical syndromes
- (vii) Strong relation to at least 2 other species of soil bacteria assigned to genus *Listeria*
- (viii) Metabolic properties

The already mentioned ability to grow at low temperatures has even been used by GRAY *et al.* in 1958 [*] to enrich listeriae in strongly contaminated material. This so-called cold enrichment method is still the best means for isolation of the pathogen from faecal matter, soil specimens and vegetable waste. Although highly sensitive to acidity, *L. monocytogenes* is resistant to high concentration of salt, alkali and also to desiccation which allows its survival under natural conditions in soil, on decayed matter, on wood, etc. This

explains the world-wide distribution of *Listeria* irrespective of climate, and it may be the main reason why so many animal species are endangered. The psychrophilic properties of *L. monocytogenes* have been substantiated further by WILKINS *et al.* [12].

The occurrence of this species outside the body has repeatedly been proved. SEELIGER *et al.* [13] cultured *Listeria* from soil specimens of a garden which was owned by a couple who both excreted listeriae with their faeces, and the woman had a listeric abortion. In connection with this observation, listeriae were also isolated from fertilizer made from non-pasteurized sludge.

OLSUFJEV (personal communication, 1964) reported on the isolation of *Listeria* from river and waste water in the Soviet Union, and subsequently WELSHIMER [14] identified the organism on decaying plant material, corn leaves and soil specimens obtained from different farms in Virginia, U.S.A. TRAIN [15] in the German Democratic Republic suspected that soil be perhaps the main habitat of *Listeria* and consequently classified these organisms primarily with the geophilic species.

Between 1969 and 1971, our laboratory reinvestigated and typed serologically a total of 698 different strains of *L. monocytogenes*, 51 of which were isolated from soil, dust, decaying vegetable matter, etc., mainly in Germany, occasionally in other countries (Table III).

Table III

Origin of L. monocytogenes strains as submitted to, and typed serologically by, the author (1969–1971)

Year	Human	Animal	Others
1969	171	59	4 Milk 2 Sewage 3 Vegetable matter 1 Kitchen
1970	88	138	3 Silage 1 Soil 1 Straw
1971	99	92	2 Silage 1 Maize fodder 20 Vegetable matter 13 Soil
Total	358	289	51

This leads to the question how the soil or plant material is contaminated with *Listeria* and whether these organisms are primary or secondary inhabitants of plants and soil. Interestingly enough NAKAHAMA in 1940 [16] described in Japan a plant parasitizing species which he named *Listeria hibiscusliquesfaciens*. A comparison of the reported biochemical features with those of *L. monocytogenes* (SEELIGER, unpublished results, 1965) would, however, exclude this species as belonging to the genus *Listeria*. On the other hand, at least 2 new species of *Listeria*, e.g. *Listeria grayi* and *Listeria murrayi* have in the past few years been isolated from faecal specimens of chinchillas and from soils in Denmark and the U.S.A. [17, 18]. The latter 2 species are non-pathogenic to animals and have a similar G : C ratio as *L. monocytogenes* with which they share partial O-antigens.

The association of *Listeria* with its possible occurrence in faecal matter had already been proposed in 1963 by E. G. D. MURRAY during the 2nd International Symposium on Listeric Infection held in Bozeman, Montana.

In spite of numerous attempts to culture the organism from faecal specimens, a positive result was obtained only occasionally, and only after prolonged storage of the specimens in the refrigerator. With the development of better media for transport and selective enrichment of *Listeria*, the number of positive isolations of *L. monocytogenes* from faecal specimens of slaughterhouse workers [17, 19–21], of healthy and sick animals [22, 23] and of healthy pregnant women [20, 24, 25] has been steadily increasing. Surprisingly, some of female listeria carriers had delivered healthy babies before positive findings were obtained after 2–3 months cold enrichment.

In these instances observed in Germany, no chemotherapy or chemoprophylaxis was applied, and yet the carrier stage was not followed by subsequent infection of the foetus.

This would indicate that occurrence of listeriae in low numbers may be more frequent in the intestinal tract of pregnant than was believed previously, and furthermore that such a carrier state must not necessarily be followed by diaplacental passage of the organism to the foetus. Thus, the pathogenesis of diaplacental infection by *Listeria* becomes even more mysterious.

At present, typical *L. monocytogenes* (i.e. strains with beta-haemolysis) are isolated at our laboratory at a rate of 1–2% from faecal specimens obtained mainly from women belonging to both groups, i.e. suspected cases of listeriosis and healthy individuals participating in a prospective study. Occasional poorly haemolytic strains isolated from faecal specimens are, however, sometimes less virulent to mice than those from infected tissues.

In two communications from the laboratory of KAMPELMACHER in Holland [26, 27] and personal communication, 1970, sensational findings were reported in that by means of a simple enrichment and selective culture positive findings have been obtained from up to 60% of human faecal specimens of

healthy pregnant. KAMPELMACHER and VAN NOORLE JANSEN [28] state that in 20 out of 26 laboratory workers (77%) and in 16 out of 26 office workers (61.5%) *L. monocytogenes* was isolated from faecal specimens. Interestingly enough, almost 50% of the strains isolated from faeces of both groups of healthy individuals proved serologically atypical (i.e. not belonging to the classical serotypes) in the agglutination test with absorbed sera and only 17 out of a total of 52 strains could be identified as serotypes 1 and 4b of *L. monocytogenes*. All chick embryos infected with non-haemolytic strains survived the experimental infection.

The isolation rate from faecal specimens of various selected groups in Hungary ranged between 1.8 and 4.2% with an average of 3.5% [20].

The findings so far obtained in 4 research projects performed in Denmark, Holland, Hungary and Western Germany would thus indicate that *L. monocytogenes* may be a resident of the intestinal tract and more or less a "normal" faecal bacterium with potentially pathogenic properties. This new concept allows to explain the widespread prevalence of listeric antibodies among the human population in the absence of clinical disease. It would also offer a satisfactory reason why in the majority of the sporadic or epidemic human infections the search for an assumed exogenic source has failed.

Similarly, faecal matter and soil may be a more important source of listeric infections in animals than any other pathway.

The use of potassium thiocyanate as advocated by LEHNERT [29] in Eastern Germany and by KRAMER and JONES [30] in England has likewise contributed to a higher isolation rate of *Listeria* [25].

Some recent results are surprising and encouraging as well. For instance, FORRAY and RALOVICH [31] and RALOVICH *et al.* [21] succeeded in isolating *L. monocytogenes* from the tonsils of pigs, and HÖHNE [32] confirmed this in the Würzburg area, where 11% out of 100 tonsil specimens from healthy slaughter pigs yielded *Listeria* within 4 days when the new media were used. This high carrier rate corroborates a recent report of KWANTES and ISAAC [33] on the very high carriage rate in chickens; 57% of 35 chickens bought in Swansea for consumption were found to have *L. monocytogenes* on their surface, regardless whether the chicken were fresh or frozen.

Consequently, *L. monocytogenes* must be widely spread in nature and in most instances be considered an epiphyte rather than a parasite. As a true opportunist, however, it may become extremely dangerous under a variety of circumstances. Thus, the reservoir of *Listeria* in nature has become so vast that its presence must be suspected in a great variety of materials. In many instances the infection would be dirt-borne depending on the more or less incidental multiplication of the organisms and its meeting a susceptible host.

Much work remains to be done in this line. It is unknown whether the excretion of listeriae via faecal matter is intermittent, incidental or continued

over longer periods of time. Moreover, the quantity of listeria cells per gram of faeces is unknown. The difficulties connected with an answer to these questions should not be underestimated. The main confusing factor is the overgrowth of specimens by enterococci which have similar nutrient and growth requirements. Recently, the use of nalidixic acid [21, 28, 34] and the combined use of nalidixic acid and acriflavine [24, 35] and other acridine dyes in semisolid media has given a better chance for selective suppression of Gram-negative organisms and faecal streptococci. Respective studies in collaboration with BOCKEMÜHL *et al.* [36], HÖHNE [32] and FEINDT [37] have resulted in the elaboration of selective media which offer a better chance for isolating *Listeria* from highly contaminated materials. The combined use of nalidixic acid and acriflavine offers a rapid and reliable method for isolating colonies of *L. monocytogenes* directly from plates. In confirming the usefulness of Stuart's medium [26] for transport of *L. monocytogenes* in contaminated materials such as faeces, FEINDT [38] elaborated a combined transport-enrichment medium for this purpose. He added acriflavine neutral (10 µg/ml) or proflavine-hemisulphate (10 µg/ml) and nalidixic acid to Stuart's medium and observed a selective and pronounced multiplication of listeriae during transport. The use of this LTAN (*Listeria* Transport medium with Acriflavine and Nalidixic acid) under practical conditions led to an about 1% positive isolation rate from faecal specimens. Although this rate is not higher than the one obtained by HÖHNE [32] with transport in Stuart's medium and subsequent plating on tryptose and nalidixic acid-acriflavine media, most positive findings were obtained within 2–3 days against much longer periods with the original method. A synopsis of the methods for selective isolation is presented in Table IV and a choice of transport-enrichment fluids in Table V.

In spite of the above, one should not forget that from time to time the disease is transmitted directly from man to man or from animal to animal. The occasional increased incidence of listeriosis in new-born wards, as reported from the U.S.A. and Israel, would suggest that the agent may be

Table IV

*Synopsis of methods of selective isolation of L. monocytogenes
from contaminated specimens*

- (i) Cold enrichment in tryptose broth at +4°C (subculturing on blood or tryptose agar at weekly or monthly intervals up to 6 months)
- (ii) Direct plating of homogenized suspension of specimen or subculture from liquid transport (or enrichment) medium to
 - (a) blood agar containing nalidixic acid, incubation for 2–4 days at 37°C; or
 - (b) tryptose agar containing nalidixic acid and acriflavine (neutral); incubation for 2–4 days at 37°C, daily reading of plates using the oblique translucent light technique

Table V

Synopsis of media used for transport and/or enrichment of L. monocytogenes in contaminated specimens

- (i) Potassium thiocyanate broth: plate daily 1 to 4 days on isolation media;
- (ii) Stuart's transport medium as used for *Enterobacteriaceae*: plate 1 to 7 days on isolation media;
- (iii) Stuart's transport medium with added nalidixic acid and acriflavine neutral: plate daily 1 to 4 days on blood agar or tryptose agar

transmitted from infected amniotic fluid and the vaginal discharge to other young mothers or babies. Contaminated hospital personnel may perhaps be part of such infectious cycles.

On several instances *L. monocytogenes* has been cultured from throat swabs of midwives, and ORTEL [38] in Halle has isolated the organism from the faeces of 4 midwives. This would point to an epidemiologic factor which so far has completely been ignored.

Last not least it should be recalled that listeriae may be harboured in the genital tract of both sexes for considerable periods of time. Since the organism has also been found in the bull's sperm, routine bacteriological control of this material has been introduced in some countries after the transmission of *Listeria* by artificial insemination had been proved. Whether the disease may be transmitted by normal copulation is, however, uncertain.

Some of the discrepancies in the different isolation rates as observed by different groups of workers need further clarification as to the bacteriological criteria for the recognition of *L. monocytogenes*. In view of the fact that occasionally non-haemolytic, serologically degraded strains have proved non-pathogenic to experimental animals, the question must be raised whether they are true *L. monocytogenes* or a related organism of less importance.

Summarizing old and new findings, listeriosis can no longer be regarded as a zoonosis or an anthroponosis *per se*. Although it may be transmitted by any species and between different species including *Homo sapiens*, exogenous sources of infection may be the decisive epidemiologic factor. Thus, contaminated fodder, listeria-containing food, listeric soil and dirt may provoke infections and even epidemics. Air-borne infection must also be considered. Thereby listeriosis assumes many characters of a geonosis.

The uptake of *L. monocytogenes* seems to result in most cases in a transient infection of the mucous membranes of the throat and of the intestinal tract without clinically recognizable symptoms, but with intermittent excretion *per vias naturales*. This leads to a potential danger, be it by endogenous infection or be it by exogenous transmission through direct contact, fomites or food, particularly in periods of increased susceptibility. These occur in progressed age, during consuming illness, during immune-suppressive therapy and administration of antimetabolic drugs.

The acceptance of an endogenous source of infection in addition to exogenous possibilities would explain why in so many cases the search for the origin of the infection remained obscure, and also why the postulated infectious chain from animals to man could be proved only exceptionally.

The particular difficulties of the application of prophylactic measures become self-explaining if one subscribes to the above concept. The need of an effective interruption of infectious chains can be fulfilled only at a modest scale. None of the usual prophylactic measures will effectively control the potential risk of endogenous infection. This can at best be reduced by rapid bacteriological diagnosis on the slightest suspicion of listeriosis which is fortunately sensitive to an early instituted and well planned antibiotic therapy.

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LISTERIA MONOCYTOGENES: SOME BIOCHEMICAL AND SEROLOGICAL ASPECTS

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Before doing biochemical or serological work with strains of *Listeria monocytogenes*, it is necessary to isolate the organism in pure culture.

For this purpose I like to use not only the normal media, but also the medium of McBRIDE and GIRARD [1]. This medium consists of an enrichment broth and a selective medium.

Enrichment broth. Tryptose phosphate broth (Difco) rehydrated and autoclaved at 15 pounds pressure for 15 min with added nitrofurantoin 1 : 10,000 to a final concentration of 1 : 100,000.

Selective medium. Phenyl ethanol agar base (Difco) containing 1% tryptose, 0.3% Bacto beef-extract, 0.5% sodium chloride, 1.5% Bacto agar and 0.25% phenyl ethanol. To this is added 0.05% lithium chloride, 1% glycine and 5% blood.

Procedure. The specimen to be cultured is inoculated in the enrichment broth and incubated at 37°C for 48 hours. Subinoculation is then made by streaking onto the selective solid medium and incubating again at 37°C for 24 to 48 hrs.

With this medium *L. monocytogenes* can be isolated from mixed cultures. There is no growth of Gram-negative organisms, but *Bacillus subtilis* and *Streptococcus faecalis* may grow. *Listeria* produces grey colonies showing no zone of haemolysis. I have tried several other selective media such as the medium of BEERENS and TAHON-CASTEL [2] with nalidixic acid, but the medium of McBRIDE and GIRARD [1] gave the best results.

Some specimens, especially faeces, require refrigeration at 4°C in tryptose phosphate broth before plating. It may sometimes take more than a year before it is possible to isolate *L. monocytogenes*.

After isolation of the organism in pure culture, the biochemical and serological properties must be determined. Biochemically, I divide my strains in 4 groups:

- (1) strains with partial β -haemolysis after 24 hrs.
- (2) strains with strong β -haemolysis after 24 hrs.

(3) strains without β -haemolysis after 24 hrs.

(4) strains without β -haemolysis, but which acidify mannitol after 24 hrs.

The standard strains of PATERSON [3, 4] belong to the first group, and also our type 1/2, 3 and 4 strains. When freshly isolated they are pathogenic for mice.

To the second group belong strains of type 5, first isolated by IWANOW [5] in Bulgaria. It is especially to these strains I want to call your attention.

These strains are also pathogenic for mice. After 24 hrs they give a strong β -haemolysis on horse-blood agar but on serum agar they grow as tiny colonies and they are slow in fermenting sugar media. After 48 hrs only glucose and fructose are fermented. Serologically these strains belong to *L. monocytogenes*, but biochemically they are different. At this moment I think every laboratory can recognize strains of *L. monocytogenes*, but strains of type 5 are easily overlooked. For a long time I thought that these type 5 strains were only isolated in Bulgaria, but last year I received 5 strains from New Zealand which were typed as type 5 by the Communicable Disease Center in Atlanta, U.S.A., of which finding they asked a confirmation. It is difficult to work with strains of type 5 as they have a tendency to auto-agglutinate. The only way to obtain reliable results is by preparing fresh antigens which must be mixed in small quantities with a mixer. Ultrasonic vibration did not give better results. I could confirm the results of the CDC in that the New Zealand strains were type 5 strains. The strains were isolated from foetuses of cows and sheep.

Before the paper by IWANOW [5] has been published I had received strains isolated from aborted calves and lambs in Holland which I had not recognized as strains of *L. monocytogenes*. After receiving the type 5 strains from New Zealand I studied 12 of these strains, 10 isolated from aborted calves and 2 strains from lambs, which had been kept freeze-dried for many years. Serologically, the strains gave the same formula as the New Zealand strains and the type 5 strain of IWANOW [5].

As type 5 strains have been isolated in Bulgaria, New Zealand and the Netherlands it is likely that they also exist in other parts of the world, but they are probably not recognized as they give completely different biochemical reactions. The strains cause strong β -haemolysis on horse-blood agar, they form no acid from trehalose, maltose, xylose, dextrin and salicin. Esculin is not hydrolyzed. It is difficult to prepare a stable O antigen from these strains. As they are pathogenic for cows and sheep, they deserve more attention than they receive at this moment.

The third group contains strains which do not cause haemolysis on horse-blood agar in 24 hrs. Most of these strains are isolated from soil, faeces or vegetation. As far as I have seen these strains are not pathogenic for mice. Serologically, these strains are different from the standard strains of PATERSON [3, 4] but biochemically they resemble the *L. monocytogenes* strains.

The fourth group of strains acidifies mannitol, and does not give β -haemolysis. Serologically these strains possess a different H antigen.

Serologically we can divide the strains into many different types, but also into groups which correspond to the 4 groups mentioned above.

The first group consists of the strains called type 1 to 4 by PATERSON [3, 4]. He demonstrated the presence of both H and O antigens in 1939/40. He distinguished a total of 4 different types:

Type 1: I, II, (III)	AB
Type 2: I, II, (III)	BD
Type 3: II, IV, (III)	AB
Type 4: V, (III)	ABC

The common factor III was regarded as unimportant by PATERSON [3, 4].

SEELIGER [6] distinguished two subtypes within type 4 strains, which he called 4a and 4b.

Serological investigations by the present author [7, 8] showed that type 4 strains can be subdivided into different subtypes:

Type 4a : (III),	V,	VII,	IX	ABC
Type 4b : (III),	V,	VI		ABC
Type 4c : (III),	V,	VII		ABC
Type 4d : (III),	(V),	VI,	VIII	ABC
Type 4e : (III),	V,	VI,	VIII, IX	ABC
Type 4ab: (III),	V,	VI,	VII, IX	ABC

The types 4a and 4b corresponded to the subtypes 4a and 4b of SEELIGER [6]. It is a pity I have lost my 4e strain.

The antigenic types isolated in Michigan by GRAY were studied by us [7] in 1958. Among type 1 strains, there were strains with the formula I, II, (III) AB, but also strains with the formula I, II, (III) ABC. Type 2 strain of PATERSON had the formula I, II, (III) BD.

In type 3 strains we can also distinguish 3 different H antigens: AB, ABC and BD.

Type 1/2a : I,	II,	(III)	AB
Type 1/2b : I,	II,	(III)	ABC
Type 1/2c : I,	II,	(III)	BD
Type 3a : II,	IV,	(III)	AB
Type 3b : II,	IV,	(III)	ABC
Type 3c : II,	IV,	(III)	BD

In 1962 IWANOW [5] described type 5 strains. These strains caused abortions in sheep. They have the following formula:

Type 5: (III), (V), VI, VIII, X ABC

The strains of type 5 give a strong β -haemolysis. They belong biochemically to the second group. They are pathogenic for mice.

In the 3rd and 4th groups we find strains which do not give β -haemolysis. These strains are nearly always isolated from faeces, soil or vegetation.

To the 3rd group belong strains of type 6 with the following formula:

Type 6: (III), VII, (VIII), XI ABC

and strains of type 7:

Type 7: (III), XII XIII ABC

The strains of types 6 and 7 do not ferment mannitol and are not pathogenic for mice.

In 1966 LARSEN and SEELIGER [9] published a new type: *L. grayi*, isolated from the faeces of an apparently healthy chinchilla. The biochemical behaviour was similar to typical *L. monocytogenes* strains, but the strain was able to ferment mannitol. Serologically the strain has a completely different H antigen:

L. grayi: (III), XII, XIV E

In 1971 in the U.S.A. WELSHIMER and MEREDITH [10] isolated strains from vegetation resembling *L. monocytogenes*. The strains were mannitol-fermenting, nitrate-reducing, avirulent organisms sufficiently different from standard *L. monocytogenes* strains and *L. grayi* to propose a new species, which they called *Listeria murrayi* in honour of the late Dr. MURRAY. They found different H antigens and proposed the formula:

<i>L. grayi</i> :	(III),	XII,	XIV	FG
<i>L. grayi</i> V 1:	(III),	XII,	XIV	FGH
<i>L. murrayi</i> :	(III),	XII,	XIV	FH

Another strain was isolated by LARSEN [11] in Denmark from chicken faeces. This strain has the following formula:

Listeria W-Li 93/65: (III), V, XV ABC

Strains resembling this type are often found in human faeces, soil or vegetation, some of which have the formula: (III), V, VI, XV ABC.

A few months ago I received 4 strains isolated from cows in Yugoslavia, one strain isolated from milk, the other three from uterine mucus. These 4 strains have the following formula:

Listeria SZ 28: (III), V, VI B

This subtype can be regarded as a loss-variant of type 4b. Antiserum against these strains contained only factor B, no other factors.

As you have seen, the serology of *L. monocytogenes* is still rather confusing. For the sake of uniformity we need a reorganization of the antigenic schema.

Summarizing, I should emphasize the importance of type 5 as these strains may easily be overlooked. They grow in tiny colonies with a strong β -haemolysis on horse-blood agar in 24 hrs. Trehalose, maltose, xylose, dextrin and salicin are not fermented and esculin is not hydrolyzed. These strains which are found in different parts of the world cause abortion in cows and sheep. They may be pathogenic for other animals and man.

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LISTERIOSIS: ITS VETERINARY HYGIENIC SIGNIFICANCE IN HUNGARY

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Listeria monocytogenes is a widespread bacterium. It has been demonstrated in surface water and fodder, mainly in silage. It survives for a long time in the soil. Different animal species were found to be carriers.

It is generally accepted that for the development of listeriosis not only the presence of the bacterium, but also certain predisposing factors are necessary. Foddering seems to have the most important influence.

The disease manifests itself in animals with septicaemia, abortion and encephalitis.

Individual forms of the disease are characteristic of certain animal species, though the three manifestations are met with alternately in the same species.

Among the domestic animals in Hungary it was in sheep that Mócsy and SÁLYI [4] in 1952 first observed listeriosis. A number of outbreaks followed in sheep as well as in geese [1], pigs [2], cattle [3] and rabbits [5].

Listeric septicaemia displays symptoms practically identical with septicaemiae of other bacterial origin. This form of the disease runs a rapid course and leads to death in 2 to 3 days. Infected animals display general symptoms (fever, anorexia, depression) accompanied sometimes with diarrhoea. In such cases the clinical symptoms fail to provide a basis for the recognition of the illness. Nervous symptoms occurring in some animals may facilitate the diagnosis.

Changes found at post mortem examination refer to septicaemia: splenomegaly, haemorrhages on different mucous and serous membranes, degeneration of the myocardium and parenchymal organs. Small inflammatory and necrotic foci in the liver can be observed even at gross examination. The mucosa of the gastrointestinal tract displays catarrhal changes.

For the reliable diagnosis, bacteriological examination must be made. Isolation of the microorganism is easy from blood and parenchymal organs.

The pathological form associated with abortion is prevalent in ruminants and rabbits. Listeriosis may, however, induce sporadic and even mass abortion also in cattle and sheep. Epidemic abortion caused by listeriosis has been

observed in a large rabbit-farm. In rabbits, too, the disease may take septicaemic course; multiplication of the pathogen occurs, however, usually in the uterus. It is the abortion that draws attention to the disease. The animal grows restless, patters and urinates frequently, hunches its back and struggles. The vulva is swollen, the perineal region and udder are oedematous and one or two days later the animal aborts. Subsequently, purulent foul discharge is passed from the uterus. If the metritis is left untreated, the animal's general condition falls and death occurs with septicaemia. Often, the foetus dies and becomes mummified or macerated *in utero*.

After post mortem examination, the uterine mucosa displays the most serious changes. The mummified and macerated foetus is also detectable in the uterus which contains a purulent foul discharge. Large areas of the uterine mucosa become necrosed. Peritonitis may also occur as well as changes referring to septicaemia.

The form of the disease affecting the nervous system may occur in every animal species. If the acute septicaemia has not led to death, in the late phase of the disease changes of the nervous system may develop. This form of listeriosis is characteristic of ruminants. Septicaemia is lacking or remains latent. Correspondingly, depression, anorexia and fever are infrequent. The first striking clinical symptoms refer directly to an involvement of the nervous system. Slightly restricted movements, grinding of teeth are followed by a stiffening of the masticatory muscles and of the neck. Compulsive movements, extremital weakness and spastic states follow each other, in some cases alternately. The occasional nystagmus is also characteristic. In the late phase of the illness the animals are unable to stand up, lying on their side they make swimming movements and after four to five days of illness they usually die.

Necropsy fails to reveal striking gross changes. The histological alterations are characteristic.

In the brain, inflammatory and regressive changes are observable. The changes involve mainly the mesenchymal region of the brain, thus the vessel walls, perivascular cavities, etc., and the ectodermal brain tissue (Fig. 1).

Layers of inflammatory cells fill the walls of precapillary and post-capillary veins as well as their dilated lymph spaces, forming in this way a vascular coat. Round cells represent the bulk of the infiltration. Large lymphocytes, monocytes, histiocytes and plasma cells are also seen with a few eosinophilic and neutrophilic granulocytes among the cells of the vascular coat. Erythrocytes present in vessel walls and fibrin threads appearing among the inflammatory cells of the infiltrated vessels are indicative of a serous infiltration.

The endothelial cells of the infiltrated vessel walls display grave degeneration (Fig. 2). They are swollen, their nuclei enlarged, the chromatin substance

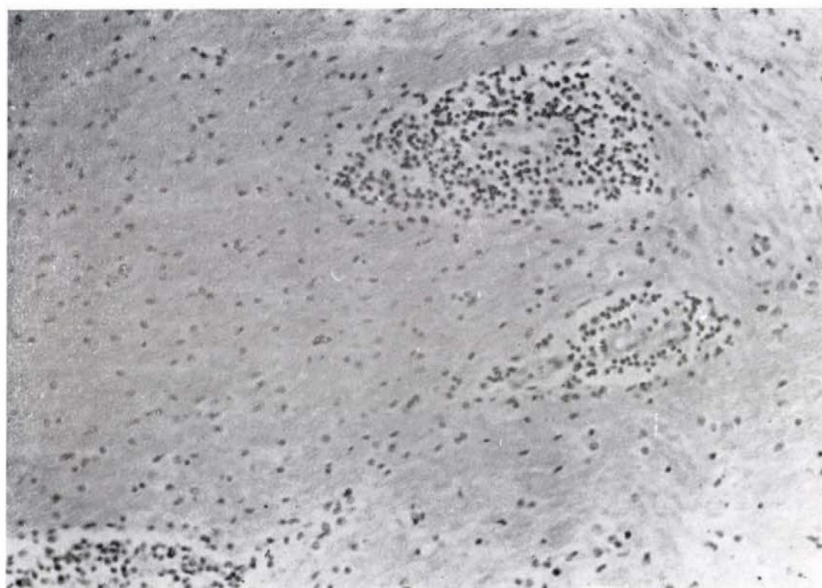


Fig. 1. Part of medulla oblongata of sheep died of listeriosis. Three small vessels with infiltrated walls (haemalaun-eosin stain, $\times 95$)

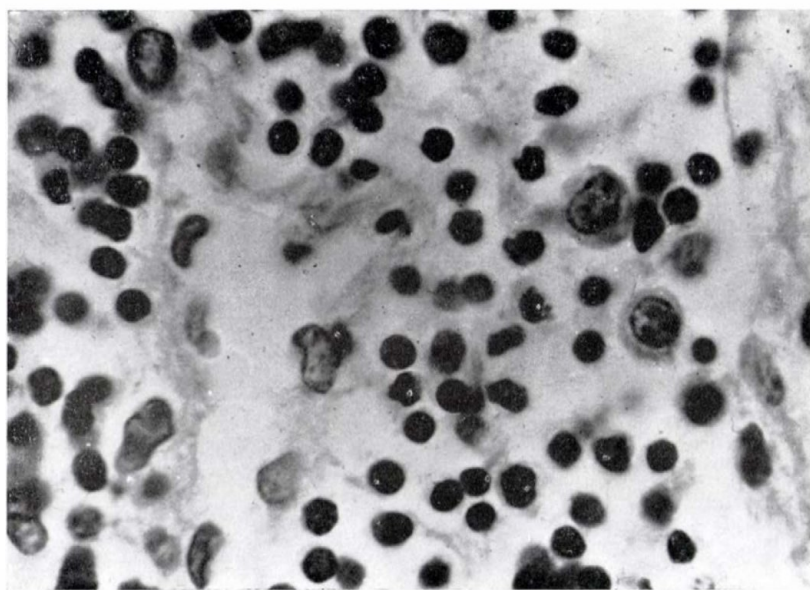


Fig. 2. Part of Figure 1. The vascular endothelium shows degeneration. Prevalently mononuclear cell infiltration in multilayer array (haemalaun-eosin stain, $\times 1200$)

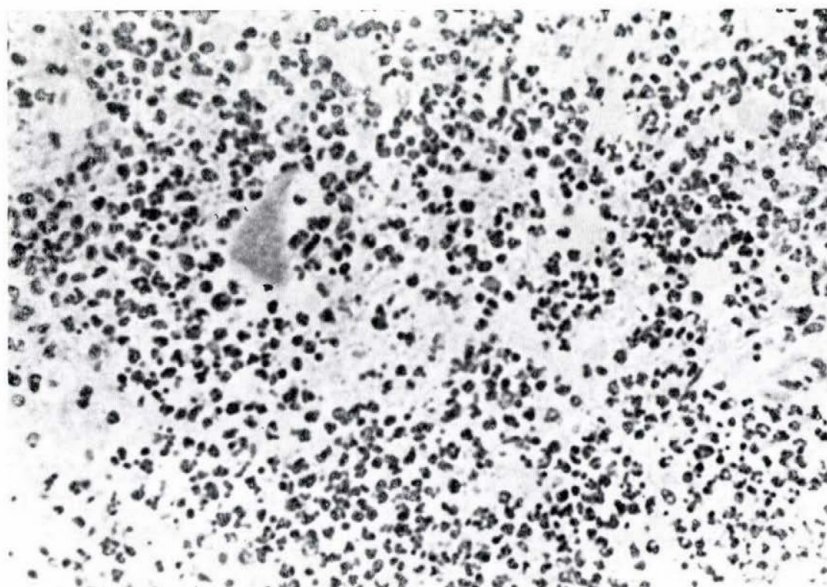


Fig. 3. Part of brain stem of sheep died of listeriosis. Degenerated nerve cell among neutrophilic granulocytes (haemalaun-eosin stain, $\times 240$)

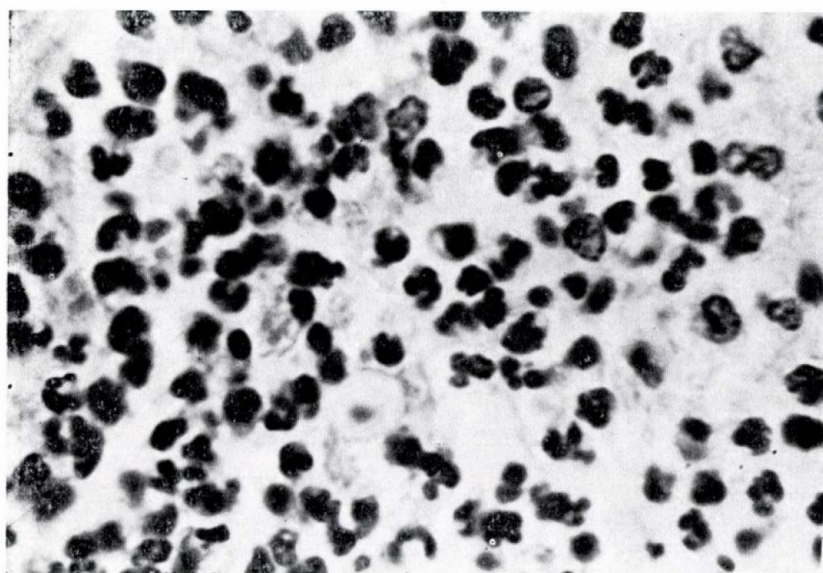


Fig. 4. Part of Figure 3. Infiltration with neutrophilic granulocytes with segmented nuclei and a few mononuclears (haemalaun-eosin stain, $\times 1200$)

shows in most cases lysis and occasionally pycnosis. Some degenerated cells are detached, the basement membrane and collagenous fibres of the vessels also display degenerative changes. The vascular coat shows the pattern characteristic of viral encephalitis.

Inflammatory changes can be observed not only in the vessels but also in the ectodermal tissue of the brain. These changes are characteristic of listeric encephalitis (Fig. 3). Focal infiltrations or, in brains displaying grave alterations, extensive cell infiltrations are present in the ectodermal tissue,



Fig. 5. Midbrain of sheep died of listeriosis. Degenerated nerve cell with blurred margins tigrolysis in the cytoplasm, the nucleus is under lysis. The surrounding ectodermal brain tissue is infiltrated prevalently by neutrophilic granulocytes (haemalaun-eosin stain, $\times 1200$)

in both the grey and the white substance of the brain stem and quadrigeminal bodies, while in the cerebellum they occur only in the white substance. The infiltrations consist almost exclusively of neutrophilic granulocytes (Fig. 4).

The infiltrated areas contain numerous degenerated or necrotized, decomposing granulocytes with disrupted nuclei. The localization of the changes is also characteristic of listeric encephalitis.

The following findings were noted by SZÉKY in 42 sheep brains in per cent of the cases: encephalitis over cortex, 28; encephalitis over cerebellum, 32; vascular cuffing in cortical white substance, 14; vascular cuffing in Ammon's horn, 15; cell infiltration in Ammon's horn, 2; vascular cuffing in

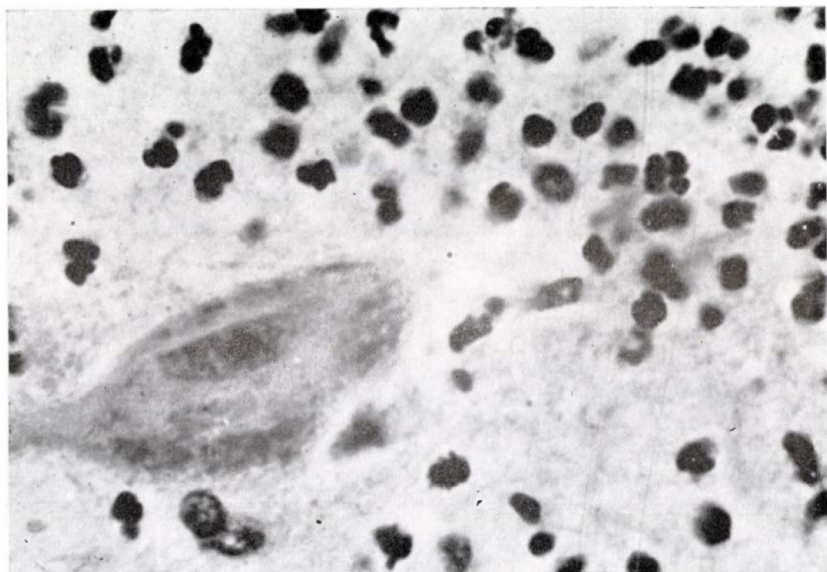


Fig. 6. Part of pons of sheep died of listeriosis. Nerve cell displays tigrolysis. The infiltration consists mainly of neutrophilic leukocytes (haemalaun-eosin stain, $\times 1200$)

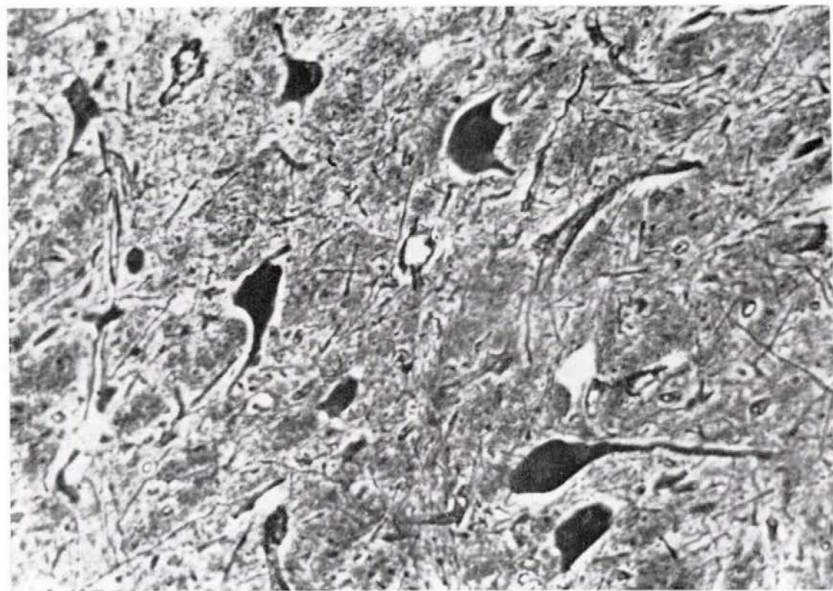


Fig. 7. Part of brain stem of sheep died of listeriosis. Bielschowsky's silver impregnation. Strong argyrophilia in degenerated nerve cells ($\times 240$)

cerebellar white substance, 30; cell infiltration in cerebellar white substance, 21; vascular cuffing in quadrigeminal bodies, 82; cell infiltration in quadrigeminal bodies, 82; vascular cuffing in pons, 100; cell infiltration in pons, 100; vascular cuffing in medulla oblongata, 100; cell infiltration in medulla oblongata, 100; vascular cuffing in cervical cord, 7; cell infiltration in cervical cord, 2; vascular cuffing in dorsal cord, 2; cell infiltration in dorsal cord, 2. Degeneration and destruction of nerve cells, nerve fibres and glia in the affected regions of the

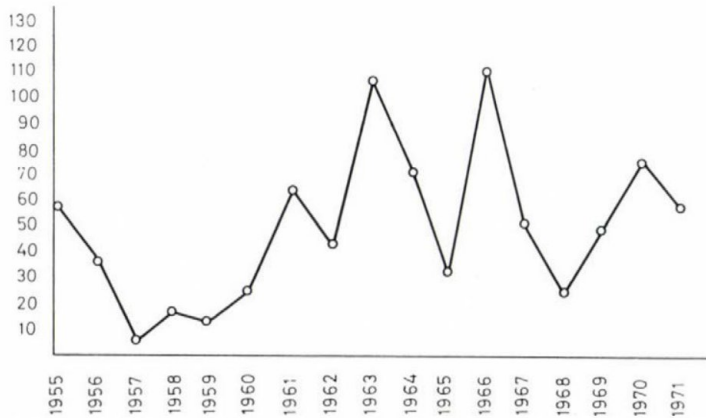


Fig. 8. Number of listeriosis cases between 1955 and 1971

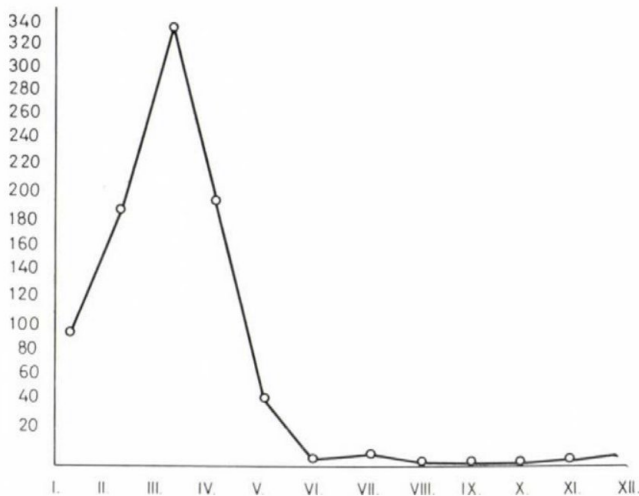


Fig. 9. Monthly distribution of listeriosis cases

brain (Figs 5—7). The brain changes were usually associated with meningitis involving mainly the envelop of the brain stem and mesencephalon.

L. monocytogenes could be cultured from the altered regions of the brain. Bacteriological examination of the parenchymal organs and blood mostly gave a negative result.

In the period from 1955 to 1971, animal listeriosis was diagnosed in 910 instances in our institute (Table I). In 90 to 95%, the agent was isolated from sheep, thus sheep listeriosis is of first economical importance in Hungary.

The incidence of listeriosis fluctuates yearly, and shows a close dependence on the season (Figs 8, 9).

Therapeutical possibilities are limited. Attempts to produce an effective vaccine have been made. The improvement of winter feeding and foddering still appears the best way, which would mean at the same time the elimination of one of the most significant predisposing factors.

Table I

Monthly incidence of Listeriosis in Hungary in the period from 1955 to 1971

Months	1955	1956	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	1967	1968	1969	1970	1971	Total
January	3	4	—	2	1	—	8	5	21	3	4	14	9	2	4	7	6	93
February	23	10	5	3	2	8	8	10	51	7	5	17	13	1	8	17	9	197
March	13	20	—	8	12	9	34	23	9	36	18	52	22	15	20	20	29	340
April	13	5	—	9	4	12	14	5	19	21	4	22	12	7	16	30	9	202
May	7	2	—	—	—	1	1	2	9	5	4	3	1	2	4	4	3	48
June	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	1
July	—	—	—	—	—	—	—	—	—	—	—	1	—	1	—	—	5	7
August	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	1
September	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	1
October	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	1
November	—	—	—	—	—	—	—	—	—	—	—	2	1	—	—	1	—	4
December	—	—	1	—	—	—	2	—	2	4	—	6	—	—	—	—	—	15
Total	59	41	6	22	19	30	67	45	111	76	36	117	58	29	53	80	61	910

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PRESENT SITUATION OF HUMAN LISTERIOSIS IN HUNGARY

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In Hungary the attention to human listeriosis was first drawn by PÁLÓCZY's review in 1956 [1]. The clinical symptoms were described by LÁSZLÓ [2]. In the same year, GERLEI [3] reported the post mortem findings of an aborted foetus, and in the author's words that paper "seemed to be the first report on a human case in Hungary". In 1957, PATAKY [4] examined the agglutination reaction of 70 mothers with a history of suspected listeriosis and in four cases found a titre of 1 : 300 and higher. FÜZI and PILLIS [5] were successful in preparing a stable listeria O antigen and, in 1962, they dealt with the differentiation of *Listeria monocytogenes* and *Erysipelothrix rhusiopathiae* [6] and with the precipitation reaction given by listeriae in egg-yolk medium [7]. Screening studies in obstetric wards carried out with the agglutination method were done by BOGNÁR *et al.* [8] in 1962. From the 12 positive cases cultivation from blood and vaginal swabs was unsuccessful. In view of the small number of cases they limited themselves to call for the need of further investigations and the introduction of more reliable methods. SZEMERÉDI [9] detailed the procedure of preparing antigens for agglutination and complement fixation tests and concluded from the investigation of 500 blood samples that an agglutination titre of 1 : 400 and a complement-fixation titre of 1 : 10 may be considered significant. FÜZI and PILLIS [10] in 1963 continued their work on the diagnosis of *L. monocytogenes*; they employed a selective blood agar containing polymyxin B and FÜZI [11] suggested a neomycin sensitivity test for the rapid diagnosis of the organism. In the same year CZEIZEL *et al.* [12] reviewed the problem of listeriosis of pregnant women. Two cases were reported, but bacteriological evidence was not presented. By applying the agglutination and complement fixation methods of SZEMERÉDI [9], DÖMÖTÖRI *et al.* [13] determined the titres in sera of 115 aborting women and 80 healthy subjects. An antibody titre of 1 : 1000 and a complement-fixation titre of 1 : 20 were found in two aborting women. *L. monocytogenes*, however, could not be cultivated either from the foetus or the placenta. JENEY [14] analyzing the current problems of the fight against anthroponoses in rural communities alluded to the question of listeriosis. In his opinion the seemingly infrequent occurrence

of listeriosis in man as contrasted to the high incidence of animal infections is to be attributed to the inadequacy of bacteriological methods and to the misidentification of the causative agent. CZEIZEL *et al.* [15] reported their serological findings in a group of 350 mothers, in which women with a history of normal deliveries, spontaneous abortions, premature births and stillbirths were equally involved. It was inferred that in 17.8% of the cases the foetal damage was attributable to *L. monocytogenes* infection. They showed that 54% of the subjects lived in villages and 19% were employed in agriculture. The authors concluded that in every 850th pregnancy one case of listeriosis is to be expected and listeriosis is responsible in 2.5% of perinatal death.

Several workers have thus attempted at establishing a relationship between abortive miscarriage and *L. monocytogenes*. However, clear-cut evidence is still lacking owing to the small number of cases, different investigation methods and the lack of cultivation results [39].

In Hungary, RODLER and SZEMES [16] were the first to record a bacteriologically confirmed case in 1966. It was a purulent meningitis and the source of infection remained obscure. Five days after the onset of symptoms the patient's serum agglutinated the live *L. monocytogenes* strain in a titre of 1 : 400. The titre reached its maximum (1 : 3200) on the 25th day of illness.

During 1967–69, the investigations on the problem of listeriosis in pregnancy and perinatal death have continued (HANCsÓK and CZEIZEL [17], DÖMÖTÖRI and KISZEL [18]), and the first bacteriologically confirmed listeriosis of a pregnant woman in Hungary was described by BODNÁR *et al.* [19]. FAZEKAS *et al.* [20] succeeded in cultivating the organism from a case of septic listeriosis of a premature infant.

The Institute of Microbiology of the University Medical School, Pécs, and the Department of Bacteriology of the National Institute of Public Health started their investigations in 1967 and 1968, respectively. They worked later in cooperation to elaborate standard methods for the bacteriological and serological diagnosis of the disease, also suitable to impart them to the laboratory network of Public Health Stations, and clarify the aetiological role of *L. monocytogenes*, to collect additional data concerning the pathomechanism of listeriosis and to estimate the epidemiological importance of the organism in Hungary.

Standardization of investigation methods was begun in 1968 by drafting a treatise that has been debated by a number of competent workers. As a result of this the standardized methods were published by MÉRŐ and RALOVICH [21] in 1969. Further epidemiological studies have been stimulated by the introduction of the new selective medium tryptaflavine–nalidixic acid–serum agar (TNSA) [22].

RALOVICH *et al.* [23] reported the cultivation of *L. monocytogenes* from the throat of a symptomless shepherd. As the person had not been ill and was

negative serologically, the case was regarded as one of symptomless carrier-ship. The authors have claimed the possibility of the circulation of *L. monocytogenes* and they stated that both human and animal spread of the disease can occur. It was established that agglutination at a titre lower than 1 : 320 was meaningless and that the agglutinin titre of normal pregnant was significantly higher than that of the control group (healthy students). They have also pointed out that persons with a history of streptococcal infection and a high AS titer showed a marked rise in listeria antibody level. The cultivation of *L. monocytogenes* was, however, unsuccessful from 142 women with a history of abnormal pregnancy and abortion.

In the first screening examinations of the faeces of slaughter-house workers we found 19 excretors out of 90 persons examined [24]. This has been followed by more extensive studies with the aim to estimate the incidence of *L. monocytogenes* in Hungary. In this program, groups of healthy slaughter-house workers, other healthy persons, pregnant and post partum women and gastroenteritis patients were involved.

L. monocytogenes was isolated in 133 cases (3.5%) out of the 3830 samples examined, in accordance with other authors who stated that the organism can often be detected in the faeces of man [25]. Most of the strains isolated were serotype 4, or belonged to the group of non-typable (NT) strains (32.4 and 33.5%, respectively). Less frequent were serotype 1/2 strains (23.3%); serotype 3 isolates were encountered in 10.5%. In Table I the strains referred to as NT type shared some antigen components characteristic of listeriae, but they could not be typed according to the accepted antigen scheme. Other workers, too, found such strains in sewage and faecal samples (e.g. DONKER-

Table I

Incidence of L. monocytogenes carriers in Hungary, 1970—1971

Groups	No. of samples examined	Serotypes				Total	
		1/2	3	4	NT*	No. of strains	Per cent
1. Healthy slaughter-house workers	2055	20	10	33	23	86	4.2
2. Other employees (healthy)	212	2	—	3	1	6	2.8
3. Pregnant and postpartum women (healthy)	1164	8	4	4	18	34	2.9
4. Enteritis patients	399	1	—	3	3	7	1.8
Total	3830	31	14	43	45	133	3.5

* Strains non-typable according to the accepted antigenic scheme

VOET [26]). *L. monocytogenes* strains of this kind were usually avirulent (no reaction after the conjunctival infection of guinea pigs, mice being not killed by intraperitoneal injection of the cultures, no beta haemolysis on blood agar). The pathogenetic role and epidemiological importance of NT strains is not clear.

We cultivated *L. monocytogenes* from the faeces of 86 healthy slaughter-house workers (an incidence of 4.2%). That the frequent occurrence of *L. monocytogenes* is correlated with direct contact with animals is suggested also by the findings of BOJSEN-MØLLER [27] who found the organism in the faeces of 55 (4.8%) out of 1150 healthy slaughter-house workers. Our screening examinations allowed to conclude that among workers dealing with animal products the number of excretors of *L. monocytogenes* is higher if the incidence of animal carriers increases. We could cultivate the organism in 22% from the faeces of the Pécs slaughter-house workers, and in the same period FORRAY [28] found a similar high frequency in the throat of swine processed in that plant. Such a high occurrence was not observed one year later at repeated examination. In other slaughter-houses in Hungary figures of 4 to 5% were obtained.

In Hungary the percentage of *L. monocytogenes* carriers among healthy individuals of other employment was 2.8%. This group comprised persons who were submitted to cultural test or subject to regular bacteriological examination of their faeces (employees in the catering trade or in children's communities, etc.). In this group, *L. monocytogenes* was detected in the faeces of a shop assistant and from that of a canning factory worker, and even a mother milk donor was found to excrete listeria. These observations indicate that in addition to milk and dairy products other foodstuffs are likely to act as means in the dissemination of the disease.

Thirty-four of 1164 pregnant and postpartum women harboured *L. monocytogenes* but no disorders of any kind were observed either in the affected mothers or in their infants. Though pregnant women with a positive bacteriological finding were treated with antibiotics, two of them still excreted the organism at the control laboratory examination carried out after delivery.

In a group of 399 gastroenteritis patients, *L. monocytogenes* was isolated in 7 cases (1.7%). Other enteropathogenic bacteria were not cultivated from the faeces of *L. monocytogenes* excretors. Similar results were reported by BOJSEN-MØLLER [27], who found the organism in 6 (1%) among 620 enteritis patients.

We may conclude from the above findings and from the pertaining literature that human carriers play an important part in the spread of human *L. monocytogenes* infection. On the other hand, the question emerges whether the organism passing into the intestinal tract exerts any harmful effect upon the host. For the present, we ignore the nature of the mechanisms which gives rise to the manifestations of listeriosis.

To approach these questions, animal infection experiments were carried out by RÁCZ *et al.* [29] at the Morphological Department of the National Institute of Public Health, Budapest. Guinea pigs were starved for 4 days, then one hour before infection calcium carbonate was given to neutralize the gastric juice, then 10 ml of a culture suspension (1.2×10^8 germs per ml) through a stomach tube. Opium was injected intraperitoneally 15 to 20 minutes after the infection and the animals were sacrificed at regular intervals. In the animals, signs of enteritis were found. Light and electron microscopy revealed that the bacteria were located intracellularly in the cells of the small intestine where they had begun to multiply before phagocytosis could have taken place. In the mucosa, soon after the infection there was a marked infiltration by polymorphonuclear leucocytes and the bacteria were readily phagocyted. The polymorphonuclear leucocytes rapidly perished and the lesion of mucosal cells was also observed. RÁCZ *et al.* [30, 31], on the basis of light and electron microscopic investigations in listeric conjunctivitis and cystitis have stated that *L. monocytogenes* as an intracellular parasitic bacterium before being phagocyted by the macrophages can multiply within the cells of the epithelial barrier through which it has gained access into the host organism. This phase of bacterial multiplication has been termed the "epithelial phase" of facultative intracellular parasitism.

On the basis of the above experimental findings we feel that under natural conditions the intestinal tract is one of the possible routes of human exposure.

The epidemiology of listeriosis is poorly understood. There is, however, a considerable amount of evidence to suggest that both human and animal carriers play an important part in the spread of the disease. GRAY and KILLINGER [32] think that it is not clear whether human listeriosis was due chiefly to a direct contact with ill animals or animal symptomless excretors. KAMPELMACHER and NOORLE JANSEN [33] concluded that direct contact with animals did not play a major part in determining the spread of listeriosis. To what extent are foodstuffs of animal origin concerned in the transfer of listeriosis is still open to investigation. The fact that *L. monocytogenes* has been detected in meat samples (neck, head, liver and heart) of deep-frozen chickens, both before and after having been chilled [34] seems to suggest that even slaughtered poultry may spread the disease. The process of sale and cooking of the meats both in households and in the catering industry, as well as prepared food can carry a risk of infection. The latter possibility is shown by the findings of RÉDEY [35] who detected *L. monocytogenes* in a food sample (dairy product).

Infected soil, water and sewage may probably also be factors in the spread of listeriosis; few studies were, however, concerned with this possibility. Cultivation of *L. monocytogenes* from infected material is difficult without

Table II
Distribution of sewage samples positive for L. monocytogenes

Site of sampling	No. of samples/ No. of samples giving positive culture		
	Total	Uncleaned sewage	Cleaned sewage
1. Soroksár	19/10	19/10	0/6
2. Mátrafüred	2/2	1/1	1/1
3. Ferihegy	6/2	2/1	4/1
4. Eger	1/0	1/0	0/0
5. Keszthely	2/1	1/1	1/0
6. László Hospital	2/0	2/0	0/0
7. Vác	3/0	1/0	2/0
8. Budakeszi	4/1	1/0	3/1
9. Szigetszentmiklós	2/2	1/1	1/1
10. Mezőkövesd	2/1	1/1	1/0
11. Visonta	1/0	1/0	0/0
12. Galyatető	1/1	1/1	0/0
13. Parád	1/0	1/0	0/0
Total	46/20	33/16	13/4

selective medium. Using the above mentioned TNSA culture medium of good selective effect DUC and MÉRŐ [36] investigated samples of sewage and surface waters with the aim of assessing the occurrence of *L. monocytogenes*. They were successful in isolating the organism from 43.5% of the sewage samples. One of the 20 samples taken from the Danube yielded a positive culture. This strain was serotype 1/2 and proved virulent. Among the strains isolated from sewage samples, 12 were serotype 1/2, 4 were serotype 4, and 6 belonged to the group of NT types.

The high percentage of positive cultures from sewage samples, and the fact that it was even detected in the water of the Danube, indicates that the pathogen is widely distributed in Hungary. With respect to the technology of slopwater cleaning and deposition as well as the use of sewage for manuring, these findings need special consideration. Surface waters as recipients are of interest as far as being exploited for water supply, utilized in irrigation and for water sports at holiday resorts.

In view of the above, it is expected that the interest in the public health importance of listeriae will increase. This was why MÉRŐ *et al.* [37] performed experiments on the resistance of *L. monocytogenes* in order to find chemical

Table III

Type distribution of L. monocytogenes strains isolated from sewage and surface water samples

Samples	No. of strains	Serotypes		
		1/2	4	Non-typable
Sewage	22*	12	4	6**
Surface water	1	1	0	0
Total	23	13	4	6

* From two samples two different serotypes were isolated

** All the 6 strains proved avirulent

and physical methods suitable for the interruption of the chain of its transmission. The knowledge of thermal death values for *L. monocytogenes* is necessary from the point of view of heat sterilization of faeces and treatment of foodstuffs, especially of milk and dairy product. Among the techniques of milk pasteurization applied in Hungary, exposure for 20 minutes at 75°C (justified experimentally by a complete kill after 15 minutes at 70°C) or for 5 minutes at 85°C has been found most satisfactory. Experiments with chemical disinfectants showed that by virtue of its bactericidal effect phenol is the best choice to kill *L. monocytogenes* in clinical samples. Chloramine is also suitable in acid solution at the usual concentration of 1:1000–2000. Bacteriostatic mercury compounds are less widely used. As indicated by experiments in progress, formaldehyde acts slowly and this limits its routine use. Hands contaminated with listeriae are best disinfected by quaternary ammonium compounds (e.g. cetylpyridinium bromide).

Data have accumulated to suggest that *L. monocytogenes* is not less common in Hungary than in other countries. It appears, moreover, that as regards the source of infection (ill animals, healthy human and animal excretors) and the factors responsible for its transmission (foodstuffs, stools, infected slopwater) the same is valid here as in other countries.

In Hungary in the last 6 years nine bacteriologically confirmed human cases of listeriosis were on record, eight of which have dated from the recent four years. The cases were found in counties Tolna, Zala, Szabolcs, Szatmár, Veszprém and in Budapest. Five of the affected persons were males and four were females. Six of the infections were lethal, five of them as a result of meningitis and one in the form of perinatal death. Newborn infants, young and aged adults were equally affected. Six times the causative organism was demonstrated in the cerebrospinal fluid, three isolations were carried out from other sources (spleen, liver, lung, placenta, umbilical blood, lochia). Seven strains were serotype 1/2, two isolates belonged to serotype 4 [38].

Table IV

*Bacteriologically confirmed cases of listeriosis in Hungary**, 1965-1971*

Site of the isolation	1965	1966	1967	1968	1969	1970	1971	Total No. of cases
County Tolna (Szekszárd)	1	—	—	1	—	—	—	2
County Szabolcs-Szatmár (Szokoly, Nagykálló)	—	—	—	2	—	—	—	2
County Veszprém (Herend)	—	—	—	—	1	—	—	1
County Zala (Lenti, Lovászi)	—	—	—	—	—	2	—	2
Budapest	—	—	—	—	—	1	1	2*
Total	1	—	—	3	1	3	1	9

* both strains were of serotype 4b

** MÉRŐ and RALOVICH [38]

We are convinced that in Hungary the incidence of listeriosis was much higher in this period. Unfortunately, clinicians are little aware of the established fact that listeriosis can be diagnosed only by isolation of the causative agent, and in most instances blood samples are sent into the laboratory for serological investigation only, though cultivation methods of adequate effectiveness are at disposal. We feel that a better awareness of the disease together with the dispelling of the above misconception concerning the diagnostic possibilities would highly stimulate the interest and contribute to an increase of detected cases.

Listeriosis is not notifiable in Hungary. The data on human incidence presented here have been gathered from case reports and personal communications and, hence, they may be incomplete. To establish with sufficient accuracy the epidemiological importance of listeriosis it seems desirable that until the obligatory notification is decreed, strains with documentation on the clinical course be sent by the isolating laboratory to the National Institute of Public Health. To receive better information, a questionnaire asking for the patients' history and the epidemiological data has been compiled.

In view of the possibility of prevention, modification of some regulations in veterinary and food hygiene seems essential. Since the organism is widely distributed in nature, in lack of other prophylactic means an educational programme directed towards a high standard of personal hygiene and hygienic information is of importance for all those who are in direct contact with animals or engaged in preparing food, employed in children's communities, among pregnant and in rural populations.

In view of the prevention against listeriosis of pregnant and against perinatal death it needs to be considered whether a screening for *L. monocytogenes* carriers should not be made compulsory. In the case of a positive finding or of fever of uncertain origin during pregnancy, administration of specific antibiotics ought to be rigorously prescribed.

The present paper is an attempt to review the situation of investigations on human listeriosis in Hungary. Naturally, we did not aspire to completeness. Preference has been given to results bearing upon the epidemiological role and public health importance of listeriosis, and to reports which in our view have dealt with aspects of practical interest. Recent studies and incomplete works have not been cited as several of them will be presented subsequently at this Symposium. As to the future trends and tasks involved in listeriosis research, they will be outlined in the closing statement of this symposium.

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ORIGINAL PAPERS

FINE STRUCTURE OF *LISTERIA MONOCYTOGENES* UNDER THE EFFECT OF AMPICILLIN

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The favourable results of ampicillin treatment in septic listeriosis of the newborn [7, 14, 15] and the good in vitro sensitivity of *Listeria monocytogenes* to this antibiotic [9, 10, 13] have prompted to study the action of ampicillin on the structure of this organism.

In our investigations we used the *L. monocytogenes* strain 338/69 (serotype 1/2 a), isolated by us from meconium of a newborn died from listeriosis. To broth cultures in the log-phase of growth were added different concentrations of ampicillin (5 μ g, 10 μ g, 50 μ g/ml) and allowed to act for $\frac{1}{2}$, 1, 2, 3, 5 hrs at room temperature. The cell sediment was fixed by osmium tetroxide, contrasted with 0.5% uranyl acetate, dehydrated in an ascending series of acetone, imbedded in Vestopal W or Durcupan ACM. Sections were made with the Reichert-Ultramicrotome OMU 2, mounted on copper grids charged with formvar and the specimens were examined in the Siemens-Elmiscope at an original magnification of 10,000–40,000 and 80 kV.

The structure of untreated listeriae, as known also from the literature [1–6], is shown in Figs 1–4. The cell is surrounded by a thick, stiff cell wall of about 310 Å. The cell wall consists of 3 layers. Two darker dense layers of nearly 30–35 Å are separated by a more clear intermediate layer. This intermediate layer measures nearly 240 Å in the represented organism. The cytoplasmic membrane is about 190 Å thick and consists of 3 layers, two dark ones measuring approximately 25–30 Å and a clear one measuring about 30 Å. The latter has a slight resemblance to the unit membrane described by ROBERTSON [11, 12]. The cytoplasm itself is filled with dense fine granules and a membrane system which different authors have termed “internal cytoplasmic membrane” [4, 5] or “plasmalemmosome” [1]. It shows the same structural elements as does the cytoplasmic membrane. The nuclear apparatus of the bacterium is characterized by a fibrillary nucleoplasm about 25 Å in thickness. Such fibrils may be long filaments of deoxyribonucleic acid.

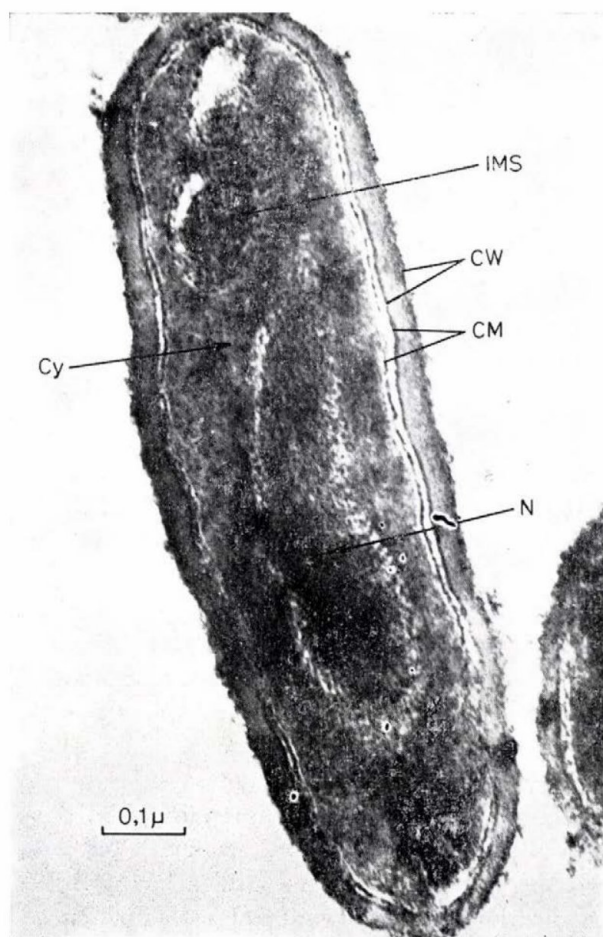


Fig. 1. Longitudinal section of an untreated listeria cell. CW = cell wall; CM = cytoplasmic membrane; IMS = internal membrane system; Cy = cytoplasm; N = nucleoid

The next figures show *L. monocytogenes* after the action of ampicillin. Electron microscopically initial changes are already apparent at concentrations of 10 $\mu\text{g}/30$ min.

Fig. 5 shows listeriae after the action of 10 μg ampicillin for 30 min. There are many elongated forms (Figs 6–8). Whereas the nuclear region is still well preserved, exposure for 1 and 2 hrs causes a destruction of the cell wall structure, a change in the cytoplasmic membrane and marked destruction in the cytoplasm. Remarkable is the development of vesicles, tubules or protuberances (Fig. 9). One can also see that the layers of the cell wall and the cytoplasmic membrane cannot be distinguished, and a strong deformation of the cell is evident (Figs 10 and 11).

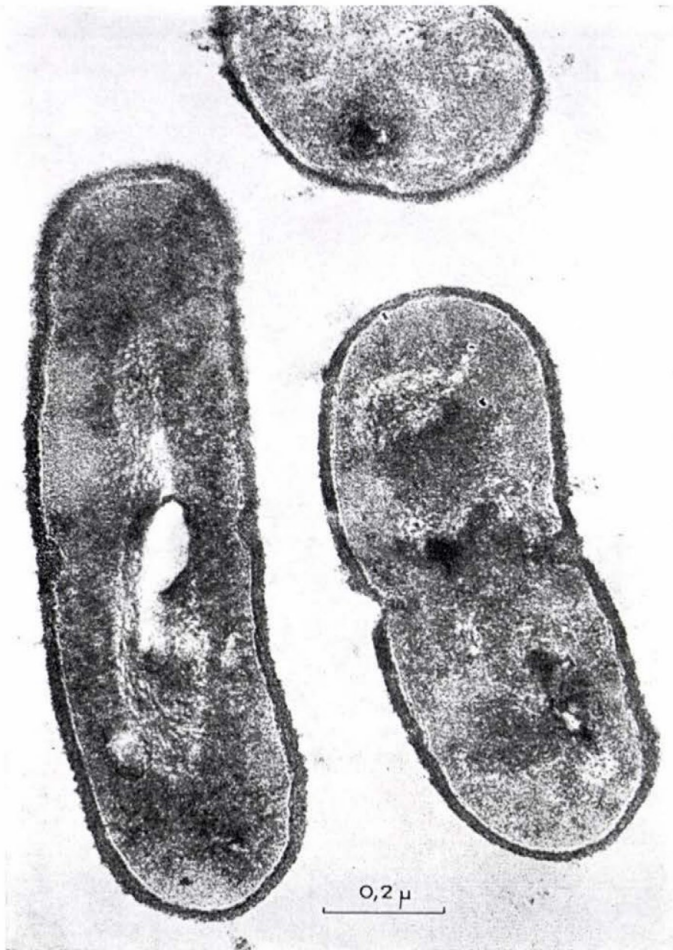


Fig. 2. Longitudinal section of untreated listeria cell. Initial phase of division

Under the action of 50 μg for 2 hrs we can see a bursting of the cell wall and of part of the cytoplasmic membrane (Figs 12—14) with swellings at one end and a flowing out of the contents. No intact cell wall can be demonstrated, and its layers and cytoplasmic membranes are destroyed and only empty membranes remain which resemble protoplasts (Fig. 15).

Thus, ampicillin rapidly inhibits the division of listeriae. First, elongated, nearly filamentous and not dividing forms occur. At higher concentrations of ampicillin acting for longer periods, remarkable destructions occur in the cytoplasm. Then we see a bursting of the cell wall and of the cytoplasmic membrane, and a loss of the cell contents and the suppression of cellular functions. Finally, at higher concentrations of ampicillin we see only empty

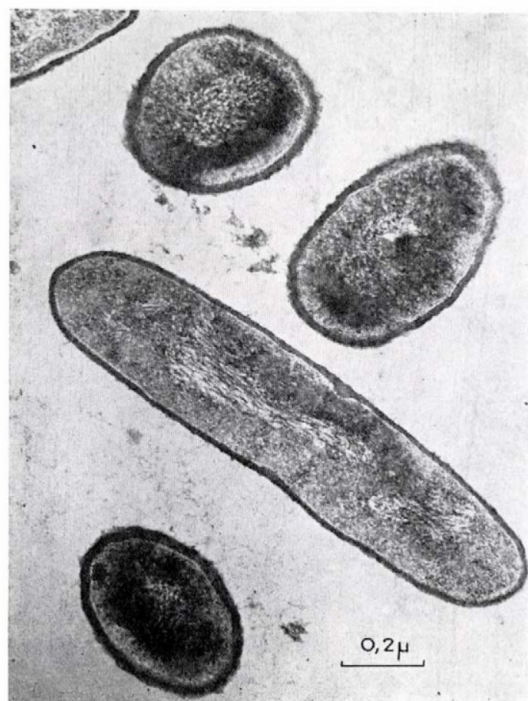


Fig. 3. Longitudinal and transverse sections of untreated listeriae. In the longitudinal section marked fibrillary nuclear structures

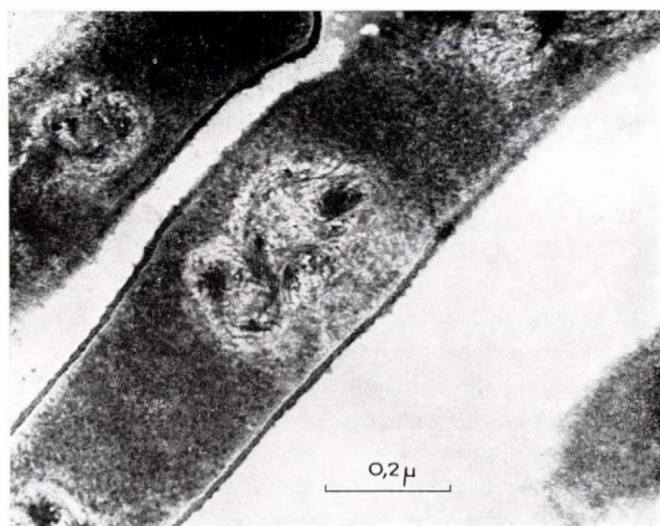


Fig. 4. Longitudinal section of listeria cell. Fibrillary nuclear structure

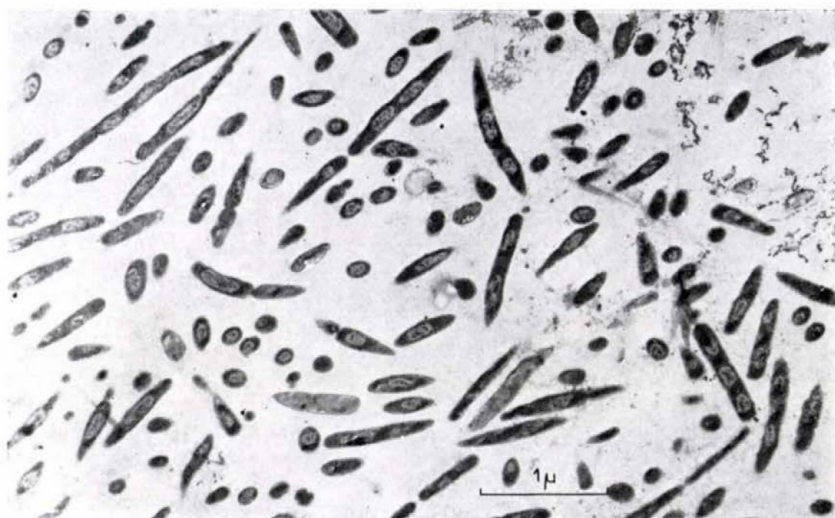


Fig. 5. *Listeriae* treated with 10 µg ampicillin for 30 min. Predominantly elongated forms



Fig. 6. *Listeria* treated with 10 µg ampicillin for 30 min. Distinctly elongated form

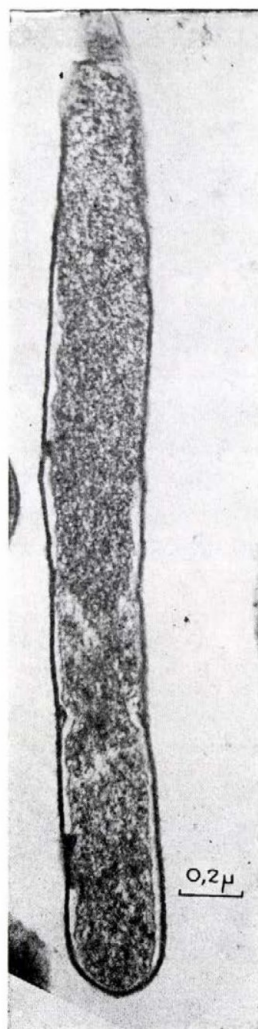


Fig. 7. *Listeria* treated with 10 μ g ampicillin for 30 min. Distinctly marked elongated form; cell wall and cytoplasmic membrane not marked

cell walls, rests of the cytoplasmic membrane and discharged clod-like cytoplasm. The results indicate that in *Listeria* ampicillin affects cellular permeability in dependence on its concentration and duration of action. The results indicate that ampicillin has a strong bactericidal action and causes changes in bacteria similar to those seen with other bactericidal antibiotics.



Fig. 8. Treatment with 10 μ g ampicillin for 30 min. Filamentous elongated form; cell wall destroyed in several places

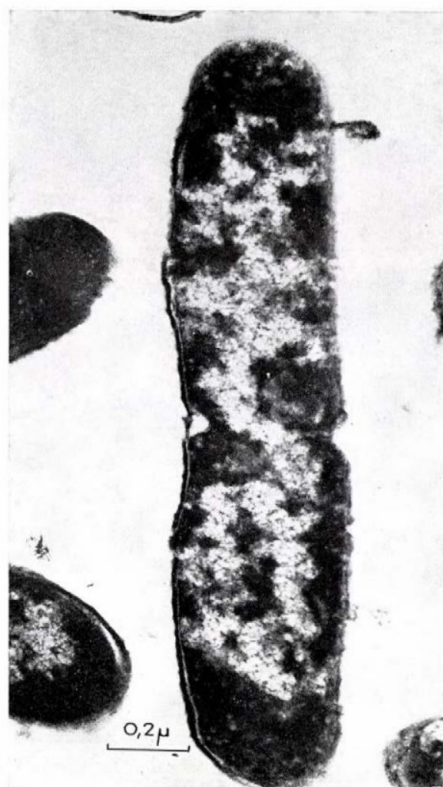


Fig. 9. Treatment with 10 μg for 2 hrs. Marked destruction of cell wall, destruction of cytoplasm; note protuberance

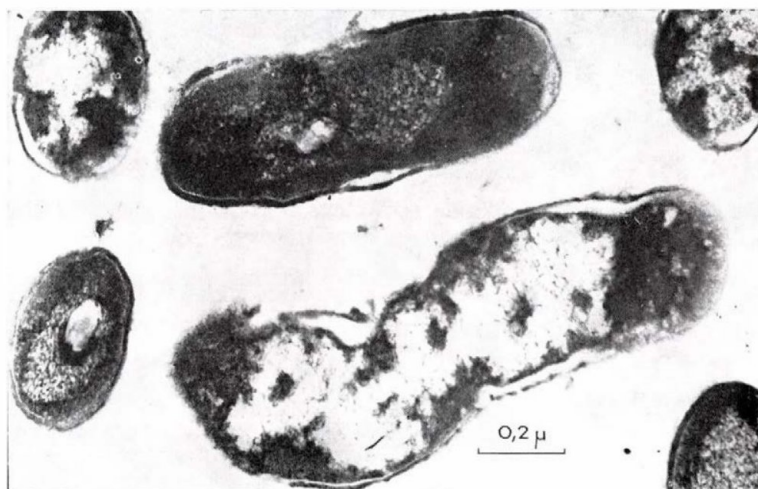


Fig. 10



Fig. 11

Figs 10 and 11. Longitudinal and transverse sections of listeriae treated with 10 µg ampicillin for 2 hrs. Structure of cell wall partially destroyed; destruction of cytoplasm in every cell; strong deformation of cells

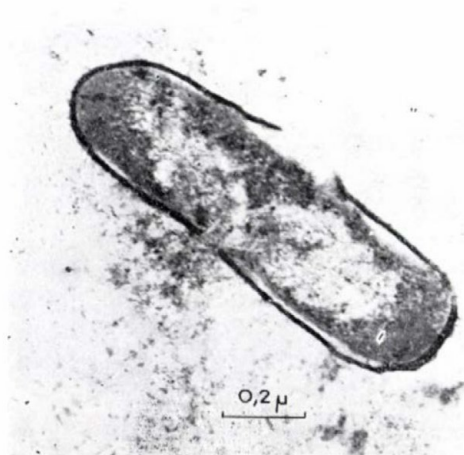


Fig. 12. Treatment with 50 µg ampicillin for 2 hrs. Bursting of cell wall in 2 places with extrusion of contents

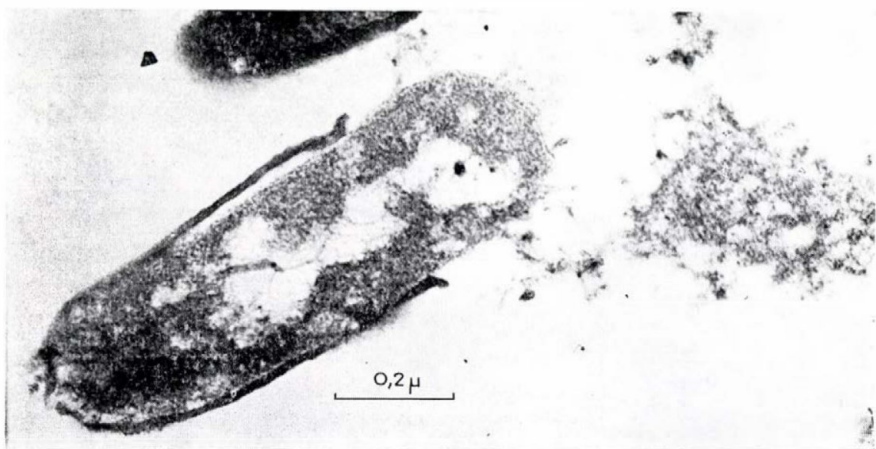


Fig. 13. Action of 50 µg ampicillin for 2 hrs. Bursting of cell wall and extrusion of contents

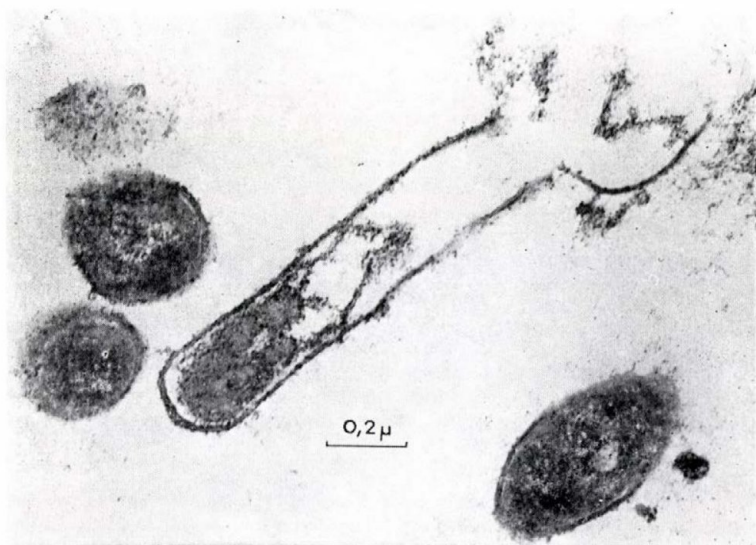


Fig. 14. Action of 50 µg ampicillin for 4 hrs. Destroyed cell with swelling at one end and extruded contents. Cell wall entirely destroyed, parts of the cytoplasmic membrane still recognizable

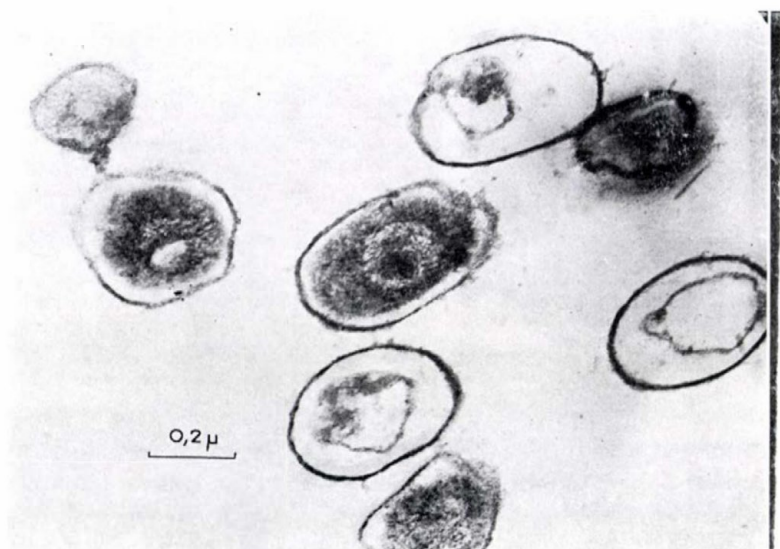


Fig. 15. Action of 50 µg ampicillin for 5 hrs. Parts of cells recognizable; structures resembling protoplasts

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BIOLOGICAL PROPERTIES OF VIRULENT AND AVIRULENT *LISTERIA MONOCYTOGENES* STRAINS

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In order to test the pathogenicity of *Listeria monocytogenes* strains, we have characterized them by markers. In this way we have examined the differences of the biological properties of virulent and avirulent *Listeria* strains.

In Table I we show the markers studied. We examined nearly 50 strains, but detailed examinations were carried out on two strains only. One of them was virulent *Listeria* strain No. 18, type 1/2a. The other was No. 10, a non-virulent strain belonging to type 4ab.

Table I

Markers of virulence of Listeria monocytogenes strains

Markers	Results	
	Virulent (1/2a type <i>Listeria</i> No. 18)	Weakly virulent (4ab type <i>Listeria</i> No. 10)
Mouse pathogenicity	0.5 ml Levinthal broth culture i.p. is lethal 10^6 germs $< LD_{50} < 10^7$ germs	0.5 ml Levinthal broth culture i.p. is not lethal 10^8 germs $< LD_{50} < 10^9$ germs
Ability to cause keratoconjunctivitis	positive reaction with more than 10^6 germs	no reaction
Chicken-embryo pathogenicity	i.v. $LD_{50} < 6 \times 10^2$ germs yolk-sac $LD_{50} < 6 \times 10^4$ germs	i.v. $LD_{50} < 3 \times 10^6$ germs yolk-sac $LD_1 < 3 \times 10^6$ germs
Beta-haemolysis	positive reaction	no haemolysis
Lipase activity	precipitation	no precipitation
Catalase activity	positive	positive

The pathogenicity of the strains was studied in mice weighing between 22 and 25 g, originating from the stock of the Animal Breeding Institute of Gödöllő, Hungary. The mice were infected intraperitoneally with 0.5 ml of Levinthal bouillon culture incubated at 37°C for 24 hrs. After inoculation,

the animals were observed for a week. Under such conditions, the virulent strains, for example strain No. 18, killed the animals in every case, while strains like No. 10 proved non-virulent.

We determined the LD_{50} of the two mentioned strains according to REED and MUENCH's method [10]. In the case of the virulent strain No. 18, this was in the magnitude of 10^6 , for avirulent strain No. 10, 10^8 – 10^9 .

If the mice were injected intraperitoneally with 0.5 ml of heat inactivated suspension of any of the two strains containing 10^9 germs per ml, no death was observed. Examining the ability of the strains to cause keratoconjunctivitis, we found that only virulent listeriae evoked a purulent inflammation of the eyes of guinea-pigs, the avirulent strains failed to cause it. To evoke a positive reaction, more than a million germs had to be smeared into the conjunctival sac.

Titration of virulence was carried out in 11 days old chicken embryos [9]. Results are demonstrated in the third heading of Table I. These data show that if injected intravenously some hundred germs of the virulent strain were sufficient for killing half of the embryos, but the LD_{50} of the weakly virulent strain amounted to more than 3 million germs. When the injection was given into the yolk-sac, in the case of the virulent strain the LD_{50} was less than 60,000 germs, while even 3 million germs of the weakly virulent strain were not sufficient to cause a single death.

During the examination of 46 *L. monocytogenes* strains, we have observed that they evoke keratoconjunctivitis, kill the mice and embryos, and cause beta type haemolysis in blood agar. Those *Listeria* strains which did not possess the mentioned capacity proved to be non-haemolyzing. These reactions [6, 7] could be repeated two years later. But we observed that the quality of the blood agar had influenced the results, therefore we suppose that perhaps this was the reason of the contradictions concerning the said phenomenon.

We have also studied the lipase activity of the strains on egg-yolk agar and L-V agar [1]. The method in the case of listeriae has first been applied by FÜZI et al. [4]. These authors have not, however, mentioned that differently growing *Listeria* strains differ from one another in respect of virulence. In connection with the examination of 46 strains, we found that listeriae were growing on egg-yolk agar in four different ways. These are demonstrated in Table II.

The strains causing no change in the medium belong to the first group; they proved to be avirulent. In the next group those weakly virulent strains were ranged which were giving rise to a slight clear zone in the medium around their colonies. The virulent strains belong to the third and fourth groups. A precipitate about their colonies in the medium is characteristic. The only difference between the strains of the 3rd and 4th groups was that the strains of group 3 formed a clear zone around their colonies besides the precipitate.

On the basis of the copper sulphate reaction, the precipitate in the medium is not a fatty acid [12].

In summary, *Listeria* strains are able to produce two kinds of substance, which in egg-yolk agar cause well-distinguishable reactions. One of the substances may be responsible for the halo around the colonies and its production does not seem to be connected with the strains' virulence. The other substance causes a precipitate; these strains also cause beta haemolysis. This means that only the virulent strains produce this latter substance. This is in accordance with the statement of GIRARD *et al.* [5]: "The supernatant

Table II
Lipase activity of Listeria monocytogenes strains
on egg-yolk agar

Groups	Change in the medium	Virulence of the strain by other markers
1	no change	weakly virulent or avirulent
2	slight clear halo	
3	precipitation and slight clear halo	virulent
4	precipitation	

filtrates from two good haemolysin producing strains also yielded good egg-yolk opacities, whereas two non-haemolysin producers were negative as was the heat inactivated haemolysin control."

Reinvestigation of the lipase activity of our strains two years later failed to give the same results as the first experiments.

We have found no correlation between the results of carbohydrate splitting and the degree of virulence of the strains [3].

In summary, the characteristic features of virulent *Listeria* strains are their mouse pathogenicity, the intense multiplicity in chicken embryo, the capacity of causing keratoconjunctivitis, beta haemolysis and the precipitation caused by lipase activity. The sensitivity to antibiotics of virulent and avirulent *Listeria* strains is also different [8]. This observation, however, as well as those showing difference in catalase [11], phosphatase [2], glutaminic acid and oxalic acid transaminase, glucose and lactic acid dehydrogenase activity of *L. monocytogenes* strains depending on the degree of virulence [11], require further studies.

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PHOSPHATASE ACTIVITY OF LISTERIA

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In a previous study, a higher phosphatase activity (PA) has been found in *Listeria monocytogenes* strains serotype 4 as compared to other serotypes [4]. Strains of serotype 4 with higher PA were more virulent than strains of other serotypes with lower PA. We have therefore studied the phosphatase production of avirulent *L. monocytogenes* strains serotype 4 in comparison with virulent strains of different serotypes. Some investigators have carried out studies of the correlation of phosphatase production and virulence of bacteria, particularly in staphylococci, but their results are not uniform [1—3, 7].

A total of 14 avirulent non-haemolytic *L. monocytogenes* strains serotype 4 of different origin has been examined. Six of these strains were isolated in the U.S.A. from vegetation and were kindly supplied by Prof. H. J. WELSHIMER [8], one strain of human origin in Hungary (kindly supplied by Dr. B. RALOVICH), 5 strains were isolated in Eastern Slovakia predominantly from the silage (kindly supplied by Dr. KOPPEL and Dr. ŠEŠEVIČKOVÁ) and 2 strains in our laboratory from clinically healthy persons. Further, we examined 4 virulent strains of the serotype 4, three virulent serotype 1 strains isolated from ill persons or from dead animals and also 6 virulent *L. monocytogenes* strains isolated from the faeces of clinically healthy persons. Most of the studied strains were isolated in various District Hygiene Centres in Czechoslovakia this year. We examined also one strain of *Listeria grayi* (kindly supplied by Dr. J. DONKER-VOET). Classification into serotype 4 was done on the basis of the strains' ability to agglutinate in factor serum V.

In order to get results comparable with previous ones concerning strains of serotypes 4 and 1, determination of PA was carried out with the technique used in the previous study [4], at acid pH as described by FISHMAN and LERNER [5] and modified for determination of phosphatase in staphylococci by KRČMÉRY [6]. A culture of *L. monocytogenes* grown on the meat-peptone agar was washed off with physiological saline and after centrifugation and washing, the suspension was standardized photometrically to the density corresponding approximately to 10^8 germs/0.1 ml. A 0.5% solution of disodium-phenylphosphate was used as substrate. The phenol split off under the effect

of phosphatase was assessed by means of the Folin phenol reagent and a 20% Na_2CO_3 solution at different intervals of incubation of a mixture of listeriae with substrate in citrate buffer (pH 5.8). Absorbance was measured by a photocolormeter Prema, type 10 E 12, at $\lambda_M = 500$ wavelength against a blank incubated without bacterial suspension in distilled water. The results to be presented were read after 48 hours of reaction when the measured values were sufficiently high. The value for phosphatase activity is given in μg of phenol released from 500 μg of sodium phenylphosphate in 1 ml citrate buffer pH 5.8 by approximately 10^8 listeriae after incubation at 37°C for 48 hours.

Table I
Phosphatase activity of *Listeria monocytogenes* strains

Avirulent strains				Virulent strains			
No.	Strain	Serotype	PA $\mu\text{gP}/48$ hrs	No.	Strain	Serotype	PA $\mu\text{gP}/48$ hrs
1	D 508	4	10.1	1	S 442	4	19.2
2	D 509	4	11.2	2	8452	4	20.0
3	S 429	4	14.1	3	4882	4	20.8
4	217	4	14.5	4	10300	4	25.0
5	S 2	4	14.5	5	322	1	11.5
6	S 9	4	15.6	6	707	1	16.0
7	S 1	4	19.6	7	687	1	17.5
8	251	4	19.9	8	220	1	5.7
9	10	4	20.1	9	230	1	7.5
10	S 12	4	21.5	10	219	1	8.5
11	D 428	4	21.5	11	234	1	9.0
12	418	4	21.5	12	222	1	9.9
13	S 11	4	21.8	13	238	1	12.8

Table I shows that there was no significant difference in phosphatase activity between avirulent and virulent strains. The avirulent strains of serotype 4 showed a similar PA as the virulent strains of *L. monocytogenes* serotype 4, or serotype 1 isolated from patients and dead animals, respectively. The only exception was an avirulent strain of serotype 4 indicated as S 8, where less than 3 μg of phenol were released in 48 hours. The test was repeated 6 times. A similar behaviour was noted with the *L. grayi* strain. There was a difference in phosphatase activity between the avirulent strains of serotype 4 and the virulent strains of serotype 1, isolated from the faeces of clinically healthy persons. While 13 avirulent strains of the serotype 4 showed PA

values between 10.1–21.8 μg of phenol, six virulent strains of serotype 1 isolated from listeria carriers had mostly a PA under 10 μg of phenol (the last 6 strains in Table I). Such a low PA value was not found in the previously studied virulent strains of serotype 1 isolated from ill persons [4]. Thus, there was a difference in PA between the strains of serotype 1 isolated from carriers and those isolated from patients, the former having a lower PA.

Thus, *L. monocytogenes* strains of serotype 4, irrespective of their virulence, have a higher phosphatase activity at acid pH than strains of serotype 1. It would be necessary to check the obtained results by examining a larger number of *L. monocytogenes* strains of serotype 1 isolated from clinically healthy persons.

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ABILITY OF *LISTERIA MONOCYTOGENES* STRAINS TO ATTACK CARBOHYDRATES AND RELATED COMPOUNDS

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The carbohydrate splitting ability of *Listeria* strains has been studied by many investigators. Besides supporting data, contradictory ones have also been published. Studying this problem, we have examined the carbohydrate splitting of 55 *Listeria* strains typed serologically, under well-controlled conditions. Furthermore, we have tested whether in this respect there was any difference between virulent and avirulent strains. We use a basic medium composed of meat extract, peptone and bromocresol purple [8]. Carbohydrate concentration was 1%, the pH was adjusted to 7.1. With the exception of starch the solutions were sterilized by filtration. The test tubes were inoculated with a 24 hr blood agar culture of the strains, and incubated at 37°C for two weeks. Results were recorded daily. In one experiment the observation was continued for 30 days. At the end of the experiments the pH of the solutions was determined with Duotest paper (Macherey-Nagel and Co., Düren, G.F.R.). When the pH was under 5.3, the result was considered positive and when it was above 6.5, negative. A positive reaction within 72 hrs was accepted as early and beyond 72 hrs as late. When the pH of the medium was between 6.5 and 5.3, or when in repeated examinations a carbohydrate was split differently by the same strain, we considered the strain to behave irregularly.

Results are shown in Table I, which demonstrates the fermentation types. The various fermentation types are represented by different numbers of the strains. For example, type A by 10, type F by 9, etc. Every strain produced acid within 3 days from D-glucose, maltose, aesculin, D-salicin, D-trehalose and laevulose. Glycerol was split by every strain, but besides early fermentation also late reactions were observed. All the strains but one split rhamnose within 72 hrs. Lactose fermentation was in most cases early positive, but late or irregular fermentation was also observed. None of the strains produced acid from adonitol, dulcitol, raffinose, mannitol, inositol and inulin. The strains — with one or two exceptions — did not split L-sorbose, D-xylose, D-sorbitol, D-arabinose and D-galactose. The starch splitting ability as well as fermentation of sucrose and melecitose of *Listeria* strains was different. There

was, however, a close similarity in the case of the two last mentioned carbohydrates. Different results were found in the case of two strains but even these were not contradictory. Namely, both strains split melecitose consequently and their sucrose fermenting ability was irregular.

While some of the strains split sucrose and melecitose late (in many cases around the 10th day), and the other strains fermented these sugars irregularly or not at all, the time of incubation was prolonged for 30 days, and the change in the number of fermenting strains was recorded. Results are demonstrated in Table II.

Table I
Splitting of carbohydrates by Listeria strains

Carbohydrates	Fermentation types and number of strains																							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	R	S	T	U	V	W	X	Y
	10	2	3	1	1	9	1	3	5	1	2	1	1	1	1	1	2	2	2	1	2	1	1	1
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aesculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Laevulose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	L	+	L	+	+	L	+	+	L	L	+	+	+	+	+	+	L	+	+	L	+	+
L-Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	I
Lactose	+	I	+	L	L	+	L	+	+	L	+	L	I	+	+	+	+	+	+	+	+	+	+	+
Adonitol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Dulcitol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Raffinose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Mannitol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inositol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Inulin	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
L-Sorbose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	L	—	—
D-Xylose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	—
D-Sorbitol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	—	—	—	—	I	I
D-Arabinose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	—	I	I	—
D-Galactose	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	I	I	—
Starch	—	—	—	—	—	—	—	L	L	L	L	L	L	L	L	—	—	L	I	I	I	I	L	—
Sucrose	L	L	L	L	—	—	—	L	L	L	L	L	—	I	I	I	I	L	L	L	L	L	I	—
Melecitose	L	L	L	L	—	—	—	L	L	L	L	L	—	L	L	I	L	L	L	L	L	L	I	—

+ positive within 72 hrs; L positive after 72 hrs; I irregular; — negative

Table II

Effect of the incubation period on the fermentation of sucrose and melecitose by 55 Listeria strains

Types of fermentation	Number of strains representing the different fermentation types	
	14 day incubation	30 day incubation
Sucrose and melecitose positive	35	39
Sucrose and melecitose negative	18	15
Sucrose positive, melecitose negative	1	1
Sucrose negative, melecitose positive	1	0

Thirty-five strains split both sugars at the end of the second week, eighteen did not ferment them and only two strains split them differently. After 30 days, 39 listeriae split both carbohydrates.

We have performed the ONPG (Koch-Light Laboratories Ltd., Colnbrook, England) reaction, as described by LOWE [3]. After 24 hrs, 80% of the strains modified the colour of the medium into a slightly yellowish one. Its intensity was far from that observed in the case of *E. coli*. It is unclear why this reaction is so weak. It seems that, unlike in enteric bacteria, there is no correlation between the result of the ONPG test and the result of lactose fermentation in some *Listeria* strains.

Finally, we compare our results with those of the literature. Up to now, every investigator has observed a positive reaction in glucose, salicin, and trehalose, and a negative result in dulcitol, raffinose, adonitol, inulin and inositol. WETZLER *et al.* [10] alone reported on a single inositol splitting strain. In spite of BURN'S [2] observation we consider laevulose among the sugars fermented by every strain. When only the classical *Listeria* strains were known, non-splitting of mannitol was a reliable marker. Since the *L. grayi* and F type strains have been isolated, the splitting of mannitol must also be taken into consideration. *Listeria* strains also seem to be homogeneous in respect of maltose and aesculin splitting. Only GRAY and KING [4] ranged these substrates among the variably fermented ones. In the case of arabinose, HARVEY and FABER [6] noticed its splitting and GRAY and KING [4] classified it as a substance fermented late or not at all. We observed an irregular behaviour for 3 strains. Rhamnose was split by most of the strains, but non-fermenting types were also observed. POTEL [7], WELSHIMER and MEREDITH [9] did not range melecitose among the differently split substances. In the case of glycerol and lactose, some authors [1, 2, 7, 9, 10] observed only fermenting types, others [4-6] reported also non-fermenting types. Most authors [1, 4-6, 10] observed both sucrose-fermenting and non-fermenting strains, but none of

Table III

Splitting of carbohydrates by *Listeria* strains

Number of strains tested	glucose	salicin	trehalose	dulcitol	raffinose	adonitol	inulin	inosito	laevulose	mannitol	maltose	aesculin	arabinose	rhamnose	melecitose	glycerol	lactose	sucrose	starch	galactose	sorbitol	xylose	sorbose	Reference
1005	+	—	—	—	—	+	—	+	+	+	+	—	D	D	L	D	L	—	—	L	L	—	—	SEELIGER [8], meat extract peptone, bromocresol purple
101	+	—	—	—	—	+	—	L	+	—	+	—	(+)	D	L	L	L	(-)	D	+	D	D	—	WETZLER <i>et al.</i> [10], Bacto purple broth
.	+	—	—	—	—	+	—	W	+	—	W	—	W	D	D	D	D	D	—	—	—	—	—	HARTWICK [5], bromocresol purple
c. 300	+	—	—	—	—	+	—	D	D	D	D	D	D	D	D	D	D	D	+	+	L	—	—	GRAY and KING [4]
4	+	—	—	—	—	—	—	(I)	+	—	—	—	—	+	L	L	—	—	+	+	L	—	—	BURN [2], Durham's peptone, Andrade's indicator
50	+	—	—	—	—	+	—	+	—	—	+	—	(L)	+	D	L	L	L	(-I)	(I)	+	(L)	—	HARVEY and FABER [6], Durham's peptone, Andrade's indicator
66	+	—	—	—	—	+	—	—	+	—	—	+	—	—	—	—	—	—	—	—	—	—	—	POTEL [7], bromocresol purple
122	+	—	—	—	—	+	—	L	+	—	+	+	+	D	D	L	L	L	D	—	D	—	D	BOJSEN-MØLLER [1], meat extract peptone, bromthymol blue
11	+	—	—	—	—	+	+	+	+	+	+	—	D	—	—	+	—	—	+	+	—	—	—	WELSHIMER and MEREDITH [9], beef extract peptone, bromocresol purple
55	+	—	—	—	—	+	—	+	+	+	+	—	(I)	(I)	D	+	+	D	D	—	—	—	—	Our results, meat extract peptone, bromocresol purple

+ positive within 72 hrs; L positive after 72 hrs; D different types; W weak acid; I irregular; — negative;
 () rare types of fermentation

them mentioned that a parallelism would exist between the splitting of sucrose and melecitose, as found by us. POTEI [7] and WELSHIMER and MEREDITH [9] obtained only negative results in connection with these two carbohydrates and we think that their observations indirectly support ours. Certain strains have the ability of splitting starch, but SEELIGER [8] and POTEI [7] found only non-fermenting strains. Concerning galactose and sorbitol, some authors reported on positive and others on negative results, and some found both fermenting and non-fermenting strains [1, 2, 4-10]. In the case of xylose,

Table IV
Diagnostic value of carbohydrate fermentation of Listeria strains

Substances according to the fermentation				
Commonly split (nearly all strains)	Not or exceptionally split	Variably split		
		Split by the majority of strains	Split by some strains	Split non characteristically
glucose	dulcitol	melecitose	mannitol	galactose
salicin	raffinose	sucrose	starch	
trehalose	adonitol	rhamnose	xylose	
laevulose	inulin	glycerol	sorbose	
aesculin	inositol	lactose		
maltose	arabinose	sorbitol		
Splitting of these substances has differential diagnostic value		Splitting of these substances may have epidemiological importance		

the results were dominantly negative, only SEELIGER [8] did not describe such a type. BOJSEN-MØLLER [1], WETZLER *et al.* [10] and ourselves examined the splitting of sorbose; most of the results were negative.

The above cited authors used different media and indicators and the concentration of carbohydrates, the way of sterilization and the period of observation were not uniform. These facts and the different biological properties of the strains should be taken into account, if an explanation of the variety of literary data is sought.

We have found no correlation between the results of carbohydrate splitting and the degree of virulence of the strains.

In our opinion those carbohydrates are useful in differential diagnostics which are uniformly or not at all split, or exception is very rare. The results connected with the splitting of the other sugars may be used only as epidemiological marker.

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BIOCHEMICAL PROPERTIES OF *LISTERIA* MONOCYTOGENES STRAINS

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For the differentiation of *Listeria*, demonstration of esterase by means of the indoxyl-butyrate paper strip method [2] as well as a modified potassium cyanide sensitivity technique have been used.

In the KCN test, the potassium cyanide was not introduced directly in the culture medium, but placed in the lid of the Petri dish. The HCN developing upon the effect of carbon dioxide and moisture diffused into the culture medium, exerting there its inhibitory effect. Applying this modification the culture media could be chosen at will and it was possible to examine the sensitivity not only of enteric bacteria but also of fastidious bacteria. Using a suitable medium, the results were evaluated after 18 to 20 hrs incubation.

The reaction was carried out by preparing a 10% KCN solution in a 4 : 6 mixture of distilled water and ethanol, the ethanol being added after dissolution. The solution could be stored in rubber stoppered bottles at room temperature for an unlimited time. The cation-adsorbing capacity of Whatman 1b paper was exhausted by keeping it in 30% NaOH for 1 to 2 minutes; subsequently, the paper was washed with distilled water until neutrality, and dried. Dried filter papers were prepared by soaking in KCN solution and cutting in 1.5×1.5 cm squares immediately after drying. The strips were then packed in cellophane and stored in glass stoppered bottles. KCN-containing papers could be stored for more than six months without any change, but after longer storage it seemed advisable to control them with known bacterial strains.

Filter papers cut into 1.2×1.2 sq.cm pieces and soaked into KCN were used without drying; they ensured the same effect as did dried strips. The KCN sensitivity test was carried out by inoculating the strains on media poured into 10 cm Petri dishes. One plate could be inoculated with 10 to 12 strains. The KCN papers were placed in the lids, 1 strip for simple agar and two strips for blood agar or eosin methylene blue medium. The Petri dishes were incubated without turning them. It is advisable to work with control strains as long as one is inexperienced in evaluation.

Indoxyl butyrate [1] appeared to be highly sensitive for the demonstration of bacterial esterase activity.

This method was performed by preparing a 0.25% solution of the substrate in ethanol. The filter paper was soaked in the solution and dried at room temperature for a few minutes. Then strips were cut and stored in glass containers in the dark.

Table I

Modified KCN reaction and indoxyl-butyrate paper strip technique in the differential diagnosis of Listeria monocytogenes

Strain	Number of strains	KCN sensitivity	Indoxyl butyrate decomposition	Motility	NO ₃ reduction	Catalase
<i>L. monocytogenes</i>	15	—	+	+	—	+
<i>E. insidiosa</i>	6	+	±	—	—	—
<i>C. diphtheriae</i>	7	—	+	—	+	+
<i>Streptococcus</i> sp.	91	—	—	—	—	—
<i>Staph. aureus</i> and <i>epidermidis</i>	72	—	+	—	+	+
<i>Bacillus</i> sp.	83	+—	+—	+—	+—	+

The reaction was carried out either by placing the substrate with the filter paper on the examined colony or by smearing the colony onto the paper strip. A few minutes after moistening of the paper, a blue colour appeared if the reaction was positive.

Observations with *Listeria monocytogenes*, *Erysipelothrix insidiosa* and *Corynebacterium diphtheriae* are presented in Table I. It is seen that *L. monocytogenes* is sensitive to KCN and decomposes indoxyl butyrate. The same behaviour was displayed by *C. diphtheriae*. The examinations were completed by including *Streptococcus*, *Staphylococcus* and aerobic spore-bearing strains. Motility, catalase activity and NO₃ reduction are also shown.

Our results demonstrate that *L. monocytogenes* strains can be differentiated from *E. insidiosa* and *Streptococcus* strains on the basis of their KCN sensitivity.

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LISTERIA MONOCYTOGENES—HAEMOLYSIN: LECITHINASE

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Listeria monocytogenes is an organism with a wide range of possible manifestations and hosts [8, 17]. It has been the subject of three international symposia (1958, 1963, 1966), a book [17] and an International Roundtable (Pécs, Hungary, 1972). Its presumptive dissemination in animals by silage [5, 6, 15], selective growth at 4°C [1] and its isolation from carriers [10] make it a possible zoonosis with food as a vector. A general study of the organism included the haemolytic aspects of the organism.

The organism produces a beta-haemolysin, which haemolyses erythrocytes of various spp. [17], is protein in nature and similar to γ -globulin [4, 7]. The amount of haemolysin is dependent on strain and growth conditions; FÜZI and PILLIS [2] and LUPPI *et al.* [13] obtained positive egg-yolk and Nagler reactions; SEELIGER [17] thought it to be a phospholipinase, yet JENKINS *et al.* [7] — using synthetic and natural triglycerides and pure lecithin — showed it to be a potent lipase rather than a phospholipase. Another problem is the influence of a soluble haemolysin on the establishment and progress of infection — e.g. it need not be equivalent to a classical exotoxin but could be an accessory virulence factor in guinea pigs, rabbits and mice [4, 14] but the route was important, highly lethal intravenously but not intraperitoneally; whilst haemolytic, DPNase and platelet damaging factors have been demonstrated too [18].

Our attempts to provide further information have included

- (1) whether the haemolysin can split phospholipids
- (2) what is its heat sensitivity
- (3) the toxicity of the haemolysin.

Most of the work was done with NCTC 5214m, a strain which caused "circling disease" in New Zealand sheep [3] and was designated type 4a [16]. The production and purification of the haemolysin followed the method of GIRARD and SBARRA [4], with the complete haemolytic unit [CHU — the reciprocal of the highest dilution giving 100% haemolysis with a 3% (v/v) erythrocyte suspension] determined after each step. Using e.g. freeze-drying and $(\text{NH}_4)_2\text{SO}_4$ precipitation it was possible to concentrate to about 4100 CHU/ml.

Table I

Relation of growth and haemolysin production

Days of incubation	3	6	9	12	15
*CHU	—	—	—	8	16
Viable count/ml	2.2×10^7	2.5×10^7	4.6×10^7	1.3×10^8	5.6×10^8

The haemolysin was doubly filtered (Swinny filter; HA 25 mm Millipore filter membrane of 0.45 micron pore size) to sterilize it. The haemolysin was standardized by titration with a carefully prepared 3% suspension of horse erythrocytes [11] to give a good calibration curve using a phosphate buffered saline (PBS) as a diluent, with 1:1 or 0.2% sodium hydrosulphite in PBS added.

Effect of temperature. The maximum production of the haemolysin (512 CHU/ml) was in TPB at 4°C when good growth at 10 days, with a low level (40 CHU/ml), went up to 256 CHU/ml at 16 days and up to 512 CHU/ml at 55 days (growth ceasing at about 24 days) — see Table I. At 30°C, maximum of 256 CHU/ml was at 6 days — maintained up to 2 months, but only 64 CHU/ml was obtained at 37°C after 2 to 4 days of incubation (see Table II).

The effect of reducing agents was marked in that e.g. addition of 0.2% NaS_2O_4 gave 256 CHU compared with 40 CHU/ml in its absence.

Table II

Production of the listerial haemolysin at two different temperatures by *L. monocytogenes* NCTC 5214m and NCTC 5214

Time, hr	30°		37°	
	Complete haemolytic unit		Complete haemolytic unit	
	NCTC 5214m	NCTC 5214	NCTC 5214m	NCTC 5214
24	128 (2.9×10^9)	8 (2.4×10^9)	32 (1.3×10^9)	8 (1.8×10^9)
48	128 (1.03×10^9)	8 (2.2×10^9)	64 (4.0×10^8)	8 (7.4×10^8)
72	128	8	64	8
96	160	10	64	8
120	160	10	—	—
144	256	10	—	—

Figures in brackets show cell count/ml

In an identical experiment CHU values were very close as presented in this table

by *L. monocytogenes* NCTC 5214m at 4°C

Days of incubation	18	24	30	38	45	55
*CHU	40	256	256	256	512	512
Viable count/ml	2.3×10^9	3.7×10^9	3.8×10^9	3.8×10^9	—	—

* Complete haemolytic unit.

Haemolysis and biological activity. Haemolytic tests of the 76 strains available showed 4 (5.3%) non-haemolytic (all Paterson type 4); 50 (65.8%) to produce a narrow zone; 13 (17.1%) to be moderate; and 9 (11.8%) to be strongly haemolytic. Using 10% egg-yolk broth in contrast to egg-yolk agar, 10 strains were positive in both and all 10 showed a moderate to positive degree of haemolysis (Table III).

Some correlation was also observed between haemolytic character and the lipase, and/or, lecithinase activity by using egg-yolk, lecithin and tributyrin (TBA) substrates. All the strongly haemolytic strains exhibited a + egg yolk reaction; produced clearing of TBA, and opacity in lecithin agar (except 2). Also in egg-yolk broth tubes a thick layer of "fat" was observed

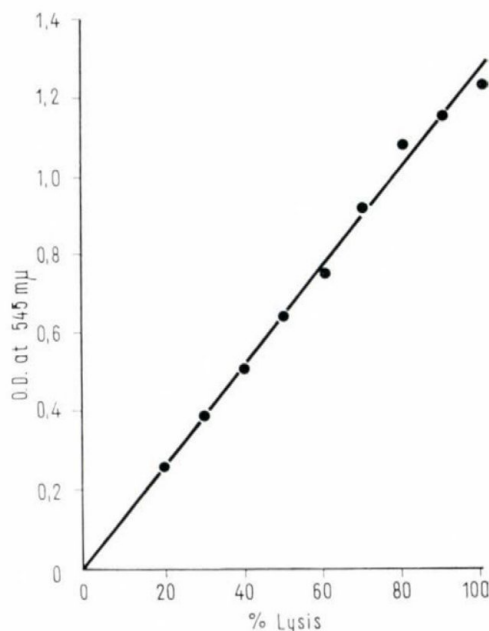


Fig. 1. Relation of optical density at 545 mμ to percentual lysis. Readings in optical density on centrifuged, undiluted supernatant

Table III
Relationship of type of haemolysis to production of opacity in egg-yolk, lecithin and clearing in tributyrin agar

Type of haemolysis on 5% horse blood agar				Opacity in 10% egg-yolk broth	Opacity in 10% egg-yolk agar	Clear zone in TBA	Opacity in 2.5% egg lecithin agar
Non-haemolytic	* Listerial like haemolysis	** Moderate degree of haemolysis	*** High degree of haemolysis				
L-991				+	—	—	—
L-1087				—	—	—	—
L-682				+	—	—	—
NCTC 10528							
	NCTC 9862			+	—	—	—
	NCTC 4883			+	—	—	—
	NCTC 5348			+	—	—	—
	L-44			+	—	—	—
	14			—	—	—	—
	La 1444			—	—	—	—
	La 1445			—	—	—	—
	L-514			+	—	—	—
	L-826			+	—	—	—
	L-1050			+	—	—	—
	L-295			+	—	—	—
	La-2465			—	—	—	—
	La 1443			—	—	—	—
	L-252			—	—	—	—
	L-994			—	—	—	—
	L-1666			—	—	—	—
	SB S/8			—	—	—	—
	SB-560			—	—	—	—
	SB-OxBr			—	—	—	—
	SB-571			—	—	—	—
	SB-692			—	—	—	—
	SB-261			+	—	—	—
	SBL-2			—	—	—	—
	SBL-3			—	—	—	—
	SBL-4			—	—	—	—
	21			—	—	—	—
	C-235			—	—	—	—
	SB-140-1			—	—	—	—
	SB-562			—	—	—	—
	SB-656			—	—	—	—
	SB-619			+	—	—	—
	SB-S/1			—	—	—	—
	SB-230			+	—	—	—
	NZ-1			—	—	—	—
	NZ-2			—	—	—	—

Table III (cont.)

Type of haemolysis on 5% horse blood agar				Opacity in 10% egg-yolk broth	Opacity in 10% egg-yolk agar	Clear zone in TBA	Opacity in 2.5% egg lecithin agar
Non-haemolytic	* Listerial like haemolysis	** Moderate degree of haemolysis	*** High degree of haemolysis				
	NZ-3			—	—	—	—
	NZ-4			+	—	—	—
	NZ-5			—	—	—	—
	NZ-6			—	—	—	—
	NZ-7			—	—	—	—
	NZ-8			—	—	—	—
	NZ-9			—	—	—	—
	NZ-10			—	—	—	—
	UCH			—	—	—	—
	Pontefract			—	—	—	—
	Shrewsbury			—	—	—	—
	COBB			+	—	—	—
	BOLDY			—	—	—	—
	SB-400			+	—	—	—
	SB-611			+	—	—	—
		L-1012		—	—	—	—
		L-268		+	+	—	—
		L-1876		+	—	—	—
		L-221		+	+	—	—
		L-1041		+	—	—	—
		L-1663		+	+	—	—
		La 2410		—	—	—	—
		L-1014		+	—	—	—
		SB-341		—	—	—	—
		SB-140-3		—	—	—	—
		SB 14012		—	—	—	—
		10		+	—	—	—
		SB 133		+	—	—	—
			NCTC 7973	+	+	+	+
			NCTC 5105	+	+	+	+
			NCTC 5214	+	+	+	+
			Li-2082	+	+	+	+
			Li-2083	+	+	+	+
			Li-2084	+	+	+	+
			Li-2085	+	+	+	+
			L-1035	+	—	+	—
			La-1205	+	—	+	—

* Very narrow zone of haemolysis around the colonies ** Slightly more haemolytic
 *** Marked haemolysis

at the surface, especially with the strongly haemolytic strains giving intense opacity. No correlation existed between serotypes, age or origin of the strains except that six of seven type 3a strains showed a + egg-yolk reaction as well as a moderate to high haemolysis. None of the narrow haemolytic zone strains gave an opacity in lecithin agar or cleared TBA.

When the erythrocytes of horse, sheep and man were examined for susceptibility to listeric (4a) haemolysin, those of man were much more resistant — those of sheep being intermediate. This is illustrated in Table IV.

Effect of Ca ions/lecithinase activity of haemolysin. The capacity of the haemolysin to produce opacity in the 10% egg-yolk saline went up markedly (see Fig. 2) with 0.1 M CaCl_2 being optimal, and the whole reaction was very rapid.

Table IV
Comparative susceptibility of horse, sheep and human erythrocytes to listerial haemolysin (type 4a)

3% suspension of erythrocytes from	Partially purified (Batch 1) CHU*	Partially purified (Batch 2) CHU	Crude (culture) CHU
Horse	4096	2048	256
Sheep	2048	1024	128
Human	1024	512	—

* Complete haemolytic unit.

The effect of Ca ions on the lecithinase activity is similar (Fig. 3), with 0.10 M CaCl_2 again optimal.

In both cases control tubes (no CaCl_2) did not show opacity until after 24 hr at 37°C or turbidity up to 90 min and a faint turbidity at 24 hr.

Effect of heat/lecithinase activity. Lecithin substrate. If the haemolysin was heated at 60°C and 80°C for 30 min there was no appreciable difference in the opacity in 2.5% lecithin (compared with control 1) but a sharp reduction (not complete elimination) occurred at 100°/15 min (compare the opacity reading Fig. 4).

Egg-yolk substrate. No effect was observed when the haemolysin was heated at 60° for 15 min but a reduction occurred with 80°C for 30 min and further heating had little effect. This was confirmed (Fig. 5) on a quantitative basis — where unheated haemolysin produced opacity within 30–60 min (and increased slowly to 210 min) but the heated haemolysin did not — but 100°C treatment gave a more intense opacity than that at 60°C or even 80°C.

All the heat treatments completely abolished the haemolytic activity (using 2048 CHU/ml haemolysin) and no regeneration occurred (up to 2 months).

Thin layer chromatography. Possible reaction products of enzyme-lecithin mixtures could be 1,2-diglycerides and phosphorylcholine and monoglyceride and fatty acids (if the haemolysin was contaminated with a lipase). Comparison with chromatographically impure controls indicates three components, one migrated the same distance as the control monoglyceride, the second could have been 1,2-diglyceride, the third fatty acids.

Antihaemolysin (lecithinase activity). Antihaemolysin was prepared from rabbits (using NCTC 5214m haemolysin at 2048 CHU; 1.0 ml + 1.0 ml Freund's adjuvant). The presence of antihaemolysin clearly affected the rate of hydrolysis of lecithin by haemolysin (Fig. 6). The inhibition was proportional to the concentration of haemolysin — the control hydrolysed in 20 min but

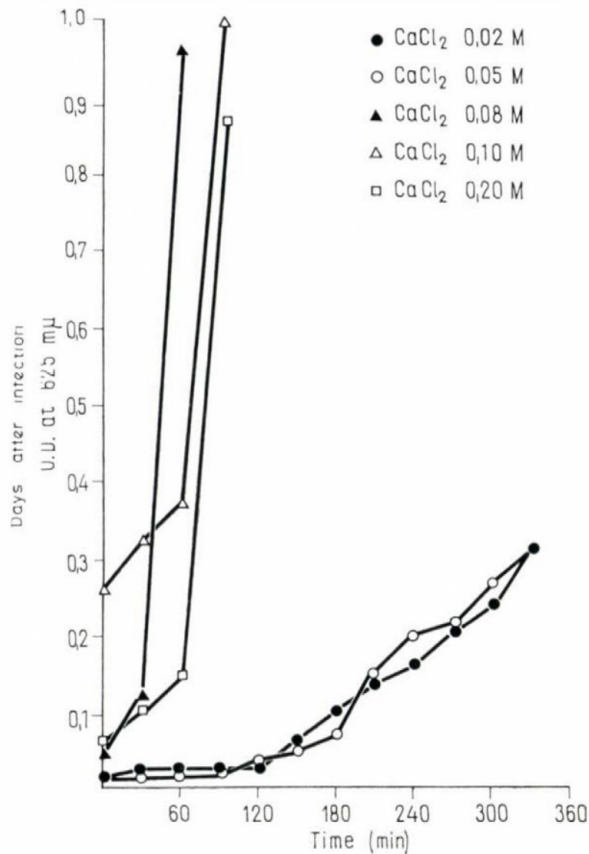


Fig. 2. Effect of Ca ions on the rate of hydrolysis of egg-yolk by the haemolysin (NCTC 5214m). Reaction mixture consists of 8.0 ml 3% NaCl + 1.0 ml egg-yolk + 0.5 ml CaCl₂ (appropriate molarity) + 0.5 ml haemolysin (1024 CHU/ml)

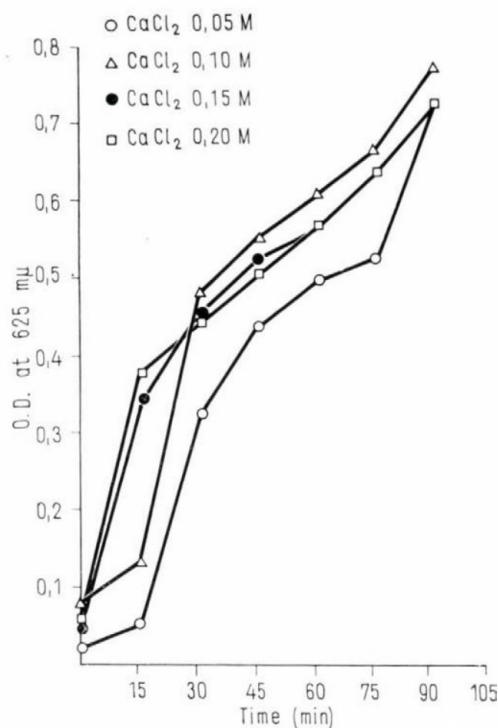


Fig. 3. Effect of Ca ions on the rate of hydrolysis of egg lecithin by the haemolysin (NCTC 5214m). Reaction mixture consists of 8.5 ml distilled water + 0.5 ml 10% lecithin + 0.5 ml CaCl₂ (appropriate molarity) + 0.5 ml haemolysin (1024 CHU/ml)

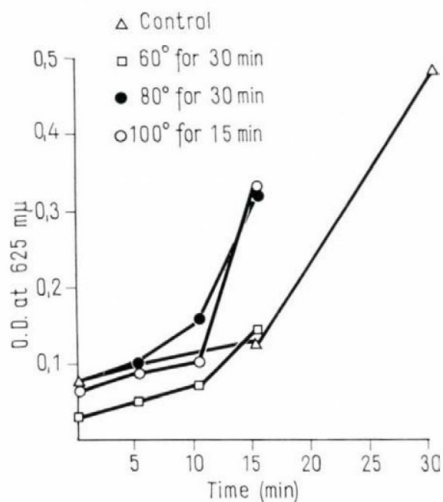


Fig. 4. Effect of heat treatment on lecithinase activity of the haemolysin (NCTC 5214m). Reaction mixture consists of 8.5 ml distilled water + 0.5 ml 10% egg lecithin + 0.5 ml CaCl₂ (0.1 M) + 0.5 ml haemolysin (1024 CHU/ml)

increasing antihaemolysin extended the time for hydrolysis to be initiated (although the lecithinase activity was not completely blocked).

Toxicity. Some toxicity studies indicated (Table V) that haemolysin produced at 4°C can be toxic. There were no obvious changes in the visceral organs of either dead mice or the already infected mice nor was any clear infection potentiation seen (as attempts have failed to isolate *Listeria* from the infected mice).

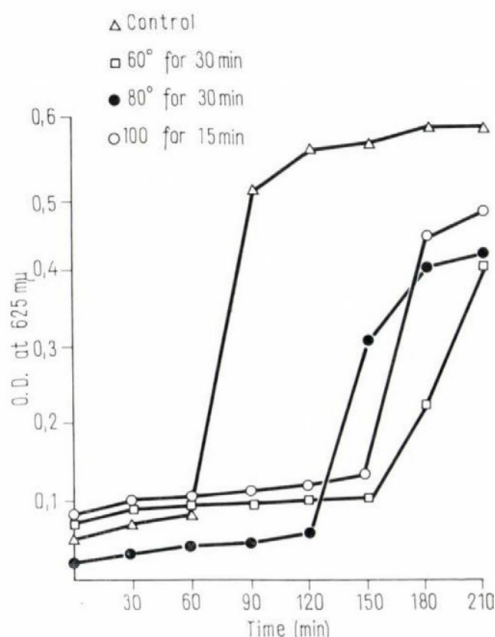


Fig. 5. Effect of heat treatment of the haemolysin (NCTC 5214m) on the production of opacity in the egg-yolk. Reaction mixture consists of 8.0 ml 3% NaCl + 1.0 ml egg-yolk + + 0.5 ml CaCl_2 (0.1 M) + 0.5 ml haemolysin (1024 CHU/ml)

It has to be borne in mind that the strain of *L. monocytogenes* NCTC 5214m is a "Champion" producer of extracellular haemolysin. Listerial haemolysin can be likened to the α -toxin (lecithinase C) of *Cl. perfringens* in its production of opacity in a lecithin medium; of reaction acceleration with Ca ions; in the possibility of 1,2-diglyceride production. But the possibility of monoglyceride and fatty acids could indicate lipolytic activity also. So the lecithinase might produce a product for subsequent lipolysis.

The effects on species of erythrocytes, however, link the haemolysin more with the β - and γ -lecithinases of *Cl. oedematiens* than the α -toxin of *Cl. perfringens*, being more active on horse and less active on sheep RBCs. Our results are in agreement with those of GIRARD and SBARRA [4] but do not exclude a possible lipase activity [7, 19].

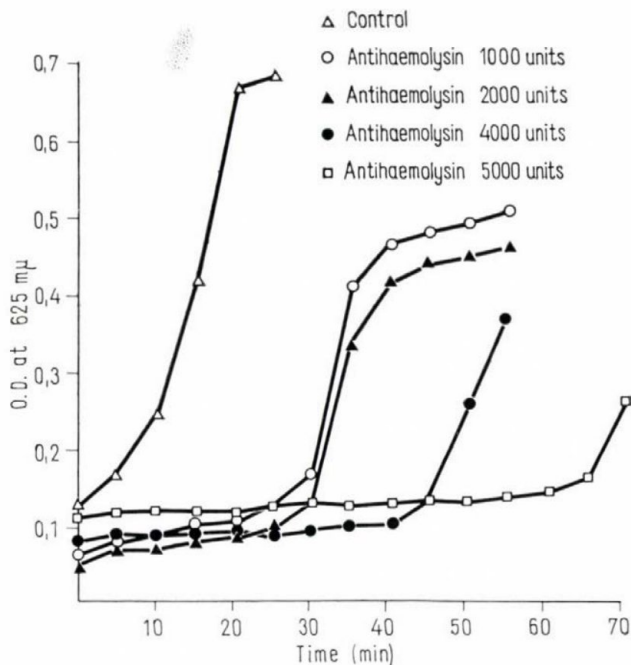


Fig. 6. Effect of specific antihaemolysin on the rate of hydrolysis of egg lecithin. Reaction mixture consists of 8.0 ml distilled water + 0.5 ml 10% lecithin + 0.5 ml CaCl_2 (0.1 M) + 1.0 ml haemolysin (32 CHU/ml) treated with antihaemolysin of appropriate strength

Table V

Results of the *in vivo* toxicity studies of listerial haemolysin (NCTC 5214m)

Haemolysin	Total number of deaths (hr)				% mortality	Attempts to re-isolate <i>Listeria</i>
	12	24	36	48		
Haemolysin produced at 4°C, injected i.p. into uninfected mice with 0.5 ml (2048 CHU)	0	2	2	3	50	
Haemolysin produced at 4°C, injected i.p. into uninfected mice with 0.25 ml (1024 CHU)	0	0	0	0	0	
Haemolysin produced at 4°C, injected i.p. into infected mice (4b; 46×10^5 cells) with 0.5 ml (2048 CHU)	3	3	3	3	50	Negative
Haemolysin produced at 4°C, injected i.p. into infected mice (4b; 4.65×10^5 cells) with 0.25 ml (1024 CHU)	0	0	0	0	0	Negative
Haemolysin produced at 30°C, injected i.p. into uninfected mice with 0.5 ml (2048 CHU)	0	0	0	0	0	
Haemolysin produced at 30°C, injected i.p. into infected mice (4b; 6.2×10^5 cells) with 0.5 ml (2048 CHU)	0	0	0	0	0	Negative

The heat inactivation studies also are in broad agreement with previous work with staphylococcal haemolysin and clostridial toxins, in terms of inactivation and partial reactivation.

From the results (Table VI) it is suggested that listeric haemolysin has some similarity with the α -toxin of *Cl. perfringens*, and also with the β - and γ -toxins of *Cl. oedematiens* and the lecithinase C of *B. cereus*. It is possible that two moieties operate — a haemolytic one, active with reducing agents, and a lecithinase one, active with Ca ions.

Table VI

Comparison between haemolysins from *L. monocytogenes*, *Cl. perfringens* (α -toxin), *Cl. oedematiens* (β & γ -toxins) and *B. cereus* (lecithinase-C)

	Haemolysin <i>L. monocytogenes</i>	Alpha-toxin <i>Cl. perfringens</i>	β/γ toxins <i>Cl. oedematiens</i>	Lecithinase-C <i>B. cereus</i>
1. Haemolytic activity	×	×	×	×
2. Lecithinase activity	×	×	×	×
3. Egg-yolk & Nagler reaction	×	×	×	×
4. Activity on egg-yolk livetin	×	×		
5. Inhibition of lecithinase & haemolytic activity by normal serum proteins	Not known	—	—	×
6. Lecithinase activity inhibited by cystein	×	×	—	
7. Lecithinase activity dependent upon Ca	×	×	×	×
8. Haemolytic activity dependent upon reducing agent	×	—	—	—
9. Haemolytic activity dependent upon Ca	—	×		×
10. Degree of haemolysis on sheep RBC	Not pronounced	Pronounced	Not pronounced	Pronounced
11. Degree of haemolysis on horse RBC	Pronounced	Not pronounced	Pronounced	Not pronounced
12. Effect on human serum & plasma	×	×	×	
13. Effect on horse & sheep serum & plasma	Weak opacity	Weak opacity		
14. Dermonecrotic effect in guinea pigs	×	×	×	×
15. Toxicity to mice	×	×	×	×
16. Anomalous behaviour to heat treatment at various temperatures	×	×	×	×
17. Hot-cold haemolysin	—	×	×	—

× = positive reaction

In terms of toxicity, *L. monocytogenes* does not compare with the exotoxins of clostridia or corynebacteria, but the haemolysin from low temperature growth was toxic to mice intraperitoneally. This is in contrast to other work [4, 7] and more akin to the intravenous studies of cultures grown at 20 or 30°C [12].

It may well be that listerial haemolysin has some role in the pathogenesis and biochemical manifestations following infection. This can be highlighted by the psychrotrophic character of the organism; the carrier status in mammals; and the possibility of food (especially meat and meat products) as a potential disseminator.

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FLAGELLATION IN VIRULENT AND AVIRULENT *LISTERIA MONOCYTOGENES* STRAINS

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SEELIGER [1] has called attention to the disagreement of the data on flagellation of various strains of *Listeria monocytogenes*.

In the past years, numerous *L. monocytogenes* strains have been isolated by us from emergency slaughtered domestic animals, from the upper respiratory tract of healthy swine and from the faeces of healthy slaughter-house workers [2–5].

Part of the *L. monocytogenes* strains isolated differed from those isolated earlier from sick animals in that the former ones failed to induce haemolysis on blood agar plates or to cause disease in rodents after parenteral infection [4, 5]. In these studies, the non-haemolytic strains exhibited definite flagellated growth and caused turbidity in Hitchens medium when incubated at 37°C for 24 hrs.

For the detection of flagellation, a semi-liquid medium prepared from papainized bovine heart was used supplemented with 0.05% sodium thioglycolate as described previously [6].

Flagellation at three different temperatures, viz. 20, 37, and 40°C, was studied in U tubes and in normal test tubes 24, 48, and 72 hrs after inoculation.

A total of 45 serologically typed *L. monocytogenes* strains was studied for flagellation, of which 26 were haemolytic, while 19 failed to induce haemolysis.

Classification of part of the strains was made by Professor SEELIGER at the Listeria Reference Laboratory, University of Medicine, Würzburg.

Studies in U tubes failed to yield convincing and reproducible results. Consequently, our findings obtained in punctured cultures will only be presented below.

As shown in the Tables, each of the strains exhibited flagellated growth at room temperature in 24 hrs. Nevertheless, two non-haemolytic strains (type 4) were also found which were non-motile at room temperature. These strains then proved minus variants that had lost their flagella.

Table I

Flagellation in avirulent strains of Listeria monocytogenes at various temperatures

No.	Type	Origin	20°C		37°C			40°C		
			24 hrs	48 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
VH 2	1/2	sewage	+		+			—	—	—
10	4ab	human faeces	+		+			—	—	—
22	4ab	swine throat	+		+			—	+	
36	4ab	swine throat	+		+			—	—	—
105	4	swine throat	+		—	—	+	—	—	—
107		swine throat	+		+			—	—	—
108	4	swine throat	+		+			—	—	—
109	4	swine throat	+		+			—	—	—
110	4	swine throat	+		+			—	+	
111	4	swine throat	+		+			—	—	—
114	4	swine throat	+		+			—	—	—
135	4	swine throat	+		+			—	—	+
136	4	swine throat	+		+			—	+	
155	4	swine throat	+		—	—	—	—	—	—
159		swine throat	+		—	+		—	—	—
MF 2	4	bovine throat	+		+			—	—	—
2862	4a	milk	+		+			—	—	—
105	4	swine throat	—	—	—	—	—	—	—	—
135	4	swine throat	—	—	—	—	—	—	—	—

Note: + = motile
 — = non-motile

After 24 hrs incubation at 37°C, virulent and avirulent strains could sharply be distinguished, since — ignoring the two minus variants — only 3 of the 19 non-haemolyzing strains studied failed to show flagellated growth and one of them failed to form flagella even after 72 hours.

On the other hand, none of the 26 haemolyzing strains studied were motile when incubated at body temperature for 24 hrs and only 7 strains were found to exhibit a less marked flagellation after 48 hrs incubation. Incubation for 72 hrs led to flagellation in 4 other strains.

After incubation at 40°C for 24 hours, neither haemolyzing nor non-haemolyzing strains formed flagella notwithstanding their vigorous growth along the puncture canal at this temperature. After 48 hrs 4, while after 72 hrs 1, viz. a total of 5 strains showed flagellated growth.

Table II

Flagellation in virulent strains of Listeria monocytogenes at various temperatures

No.	Type	Origin	20°C		37°C			40°C		
			24 hrs	48 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
15	1/2c	swine throat	+		—	—	—	—	—	—
27	1/2c	swine throat	+		—	—	—	—	—	—
89	1/2a	swine throat	+		—	+	—	—	—	—
94	1/2a	swine throat	+		—	—	—	—	—	—
105	1/2	swine throat	+		—	+		—	—	—
115	1/2	swine throat	+		—	+		—	+	
139	1/2	swine throat	+		—	+		—	—	—
156	1/2	swine throat	+		—	—	—	—	—	—
157	1/2	swine throat	+		—	+		—	—	—
164	1/2	swine throat	+		—	—	+	—	—	—
I	1/2a	rabbit	+		—	+		—	—	—
II	1/2	rabbit	+		—	—	+	—	—	—
III	1/2	rabbit	+		—	—	—	—	—	—
724	1/2	sheep brain	+		—	—	+	—	—	—
744	1/2	sheep brain	+		—	+		—	—	—
829	1/2	sheep brain	+		—	—	—	—	—	—
865	1/2	sheep brain	+		—	—	—	—	—	—
880	1/2	sheep brain	+		—	—	+	—	—	—
884	1/2	sheep brain	+		—	—	—	—	—	—
1201	1/2	sheep brain	+		—	—	—	—	—	—
1719	1/2	sheep brain	+		—	—	—	—	—	—
1056	1/2	bovine lymph node	+		—	—	—	—	—	—
2806	4b	human CSF	+		—	—	—	—	—	—
2813	4b	human haemo-culture	+		—	—	—	—	—	—
2832	4b	human CSF	+		—	—	—	—	—	—
2865	4b	human CSF	—	+	—	—	—	—	—	—

In previous studies [4, 5] a correlation was found between haemolyzing ability and the virulence of *L. monocytogenes* strains. The haemolytic strains had been isolated mainly from pathological cases while part of the *L. monocytogenes* strains isolated during a screening of healthy humans and swines failed to induce haemolysis. Freshly isolated non-haemolytic strains failed to cause infection in experimental animals [4, 5].

Later, a close correlation between flagellation and haemolyzing ability has been demonstrated. When cultivated in semi-liquid medium at different temperatures, most of the non-haemolytic avirulent strains showed flagellated growth as soon as after 24 hrs incubation at 37°C, while none of the haemolytic virulent strains was found to form flagella after 24 hrs incubation at body temperature. When cultivated at 20°C, motility was detected in both groups, while neither of the groups showed flagellation when incubated at 40°C for 24 hrs. Motility was most marked after the first 24 hrs of incubation. Though part of the strains formed flagella after 48 and 72 hrs of incubation at 37°C and a few strains also at 40°C, the majority of those from pathological human and animal cases failed to exhibit flagellated growth even after 72 hrs incubation.

It is suggested that further studies be made in order to determine the extent to which the capacity of *Listeria* to form flagella at body temperature can influence the invasion of non-haemolytic *Listeria* when introduced into human and animal organisms.

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SURVIVAL VERSUS GROWTH OF A FACULTATIVE PSYCHROTROPH

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The main sources of information about human and animal infections due to *Listeria monocytogenes* [1–3] provide evidence for the ubiquitous nature of the organism. Actual reports of human and animal infections have increased (as can be seen in the weekly summaries of the U.K. Public Health Laboratory Service 1945–65 [4]; *Brit. med. J.* for 20th December, 1969; or the U.S.A. Morbidity and Mortality Weekly Reports in March and September, 1970). These may be due to a greater number of cases *per se* or either to improved techniques of identification or, most likely, awareness of the organism as a problem.

There are difficulties in the isolation of the organism from biological specimens with the help of suitable media [5]. Such conventional techniques are time consuming, and more rapid, specific sensitive methods would be useful, such as in the fluorescent antibody technique [6, 7]. Little attention has, however, been given to the detection of the bacterium in foods of animal origin which could act as potential sources of infection [7–9]. The investigation considered to be necessary [7] could be regarded under four heads:

- (1) growth and survival of the organism in prepacked meats;
- (2) effects of temperature and packaging on growth;
- (3) growth and survival in a model isolated meat protein such as sarcoplasmic protein ("drip");
- (4) the development, standardization and application of the possibly rapid immunofluorescent technique for both rapid detection and serological identification of the organism (e.g. using artificially infected meat, milk and animal tissues).

In view psychrotropic character of the organism and the intriguing comparative results from the sarcoplasmic proteins used in this paper will emphasize these aspects.

Sterile meat preparation was based on the method of SHARP [10] using an aseptic method and normal healthy animals. The inoculum was a 30°C, 18 hr culture in 20 ml tryptose phosphate broth (TPB) diluted to contain 10^5 cells per ml, and 0.1 ml per g meat was used (10^4 cells per g). This was

injected into e.g. 225 g portions with a 7 cm needle and a portion was also spread over the surface of the sample.

The packaging materials — both gas permeable (GP) films (W. R. Grace Ltd., London) which had a permeability for water vapour of 0.6 to 0.8 g at 100% RH per ml per 100 sq.in. per 24 hr, and for oxygen of 200 ml per 24 hr per sq.m. per atmosphere differential at 20°C, and gas impermeable (GI) films — were heat sealed after the 225 g meat portions had been inoculated and aseptically inserted. They were then stored at 0 and 8°C.

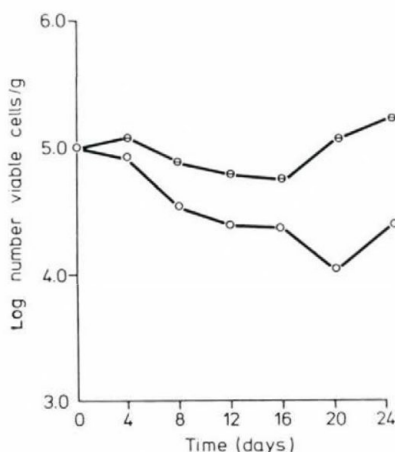


Fig. 1. Effect of gas permeable (⊖) and gas impermeable (○) films on the growth of *L. monocytogenes* (SB-692) at 0°C in sterile lamb meat

Bacteriological analysis was based on the recommendations of the International Committee on Microbiological Specifications for Foods [11]. Counting of *L. monocytogenes* (SB 692), however, used two of the selective media [5], tryptose Negram agar (TNA) and tryptose, thallous acetate, Negram agar (TTNA).

Minced meat and sausage meat as purchased locally were also used, inoculated, packed and examined in a similar way.

Isolated meat protein (sarcoplasmic protein, or "drip") from lambs and pigs was that used by PALMAS [12] in his general study of "drip" (see Table I for the physical and chemical data). It was dispensed into 2 ml and 5 ml aliquots, inoculated with 1.5×10^4 viable cells per ml and incubated at 4 and 8°C. The number of organisms assessed at intervals by using calibrated Pasteur pipettes (0.1 ml) and triplicate plates were incubated at 30°C for 48 hr.

The results for the survival and growth of *L. monocytogenes* in sterile lamb meat in GP and GI film packs at 0 and 8°C showed that the two storage temperatures had a pronounced effect in that at 0°C (Fig. 1) there was a down-

ward trend until 16 days (GP) and 20 days (GI) but no such fall occurred at 8°C (Fig. 2). In both cases, counts were lower in the GI packs.

Results for the minced and sausage meats showed that the organisms were isolable — and indeed may have increased in number — for 15 days at

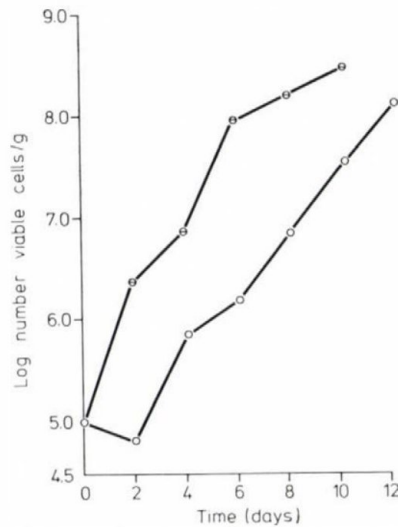


Fig. 2. Effect of gas permeable (⊖) and gas impermeable (○) films on the growth of *L. monocytogenes* at 8°C in sterile lamb meat

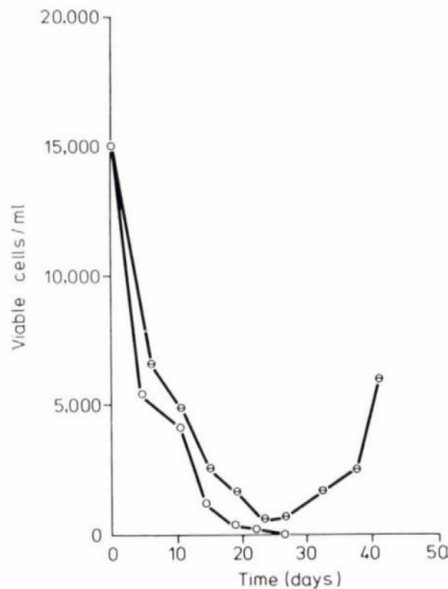


Fig. 3. Growth of *L. monocytogenes* (SB-692) at 4°C in the sarcoplasmic proteins from lamb (⊖) and pork (○)

8°C and 20 days at 4°C in contrast to e.g. control unpackaged meat at room temperature of up to 3 days. But attempts at selective counting of *Listeria* by using the two selective media and HENRY's [13] oblique illumination technique failed in face of the difficulty of clearly separating out micrococci, streptococci or other organisms present.

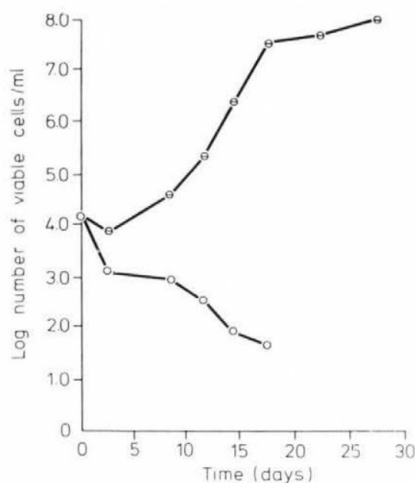


Fig. 4. Growth of *L. monocytogenes* (SB-692) at 8°C in the sarcoplasmic proteins from lamb (○) and pork (◻)

The results for the sarcoplasmic protein, however, were intriguing in that at both 4°C and 8°C in pork (Fig. 3), *Listeria* was unable to survive and slowly died off in 15 to 25 days. In sharp contrast, the lamb (Fig. 4) supported growth at both temperatures after an initial downward trend. These results were confirmed with another batch of the two drips.

Discussion

In spite of the fact that farm animals and poultry are susceptible to *Listeria*, it is difficult to indicate how much alimentary listeric infection is caused by the consumption of contaminated foods. One major obstacle is the time needed, as initial attempts to isolate from a suspected specimen may fail, though subsequent attempts — after cold incubation [14] — may be positive. *Listeria* can remain viable in the organs and meat for a long time [15, 16], so meat and meat products could be involved in the transmission of human listeriosis. Carcasses may be contaminated by slaughterhouse workers' acting as carriers [8]. Again, any subsequent heat treatment may be inadequate in that low temperature pasteurization did not kill all the *Listeria* in milk if the

number exceeded 5×10^4 [17] and multiplication was possible during low temperature storage.

Our results emphasize that two of the factors influencing the growth of *Listeria* are temperature of storage [17] and packaging [6]. The differences in the sterile meat packed in GP and GI films may be due to physical differences and CO_2 , for example, which can be produced in prepacked meat in storage [18, 19] and may be inhibitory.

The growth in "drip" is intriguing in that the two sarcoplasmic proteins differ qualitatively in their amino acid content (Table I). It could well be that the presence of lysine, serine and valine — relatively absent in the pork — is supporting growth. The nutrition of *Listeria* (and *Erysipelothrix*) has not attracted much interest since the early studies of HUTNER [20] and PORTER and PELCZAR [21] had indicated that riboflavin, biotin and haemin helped in the growth of *Listeria* — using an acid hydrolyzed vitamin-free casein salt medium. Whether pork protein lacks some factors needed for the growth and survival of *Listeria* — the death of the organism indirectly explains the much lower incidence of listeriosis in pigs compared with sheep — why, indeed, that the pig appears to be the principal host for *E. rhusiopathiae* and the sheep for *L. monocytogenes*. It could well be that further work in the nutrition of *Listeria*/*Erysipelothrix* will shed light on this susceptibility to infection.

Table I

Physical and chemical data of lamb and pork sarcoplasmic protein (longissimus dorsi)

	Lamb	Pork
pH	5.96	5.88
Protein, per cent	4	6
L-Alanine	*+	*+
L-Alanine	+	+
Arginine	*+	*+
Aspartic acid	+	+
Glutamic acid	*+	*+
Histidine	*+	*+
Lysine	*+	*—
Methionine	+	+
Phenylalanine	+	+
Serine	+	—
Threonine	—	+
Valine	+	—

* Principle amino acid according to colour intensity (Data from PALMAS, C. V.)

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EXPERIENCE WITH NALIDIXIC ACID— TRYPAFLAVINE AGAR

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Since RALOVICH *et al.* [5] had recommended the nalidixic acid–trypaflavine agar to isolate *Listeria monocytogenes* from samples contaminated by enterococci, experiments with this medium have been carried out by several authors [1, 3, 4]. In this paper we report on further experience. We compared the usefulness of this medium with that of the nalidixic acid agar. Tryptose agar (Difco) was used as basal medium. We examined samples of faeces and meconium. Samples of faeces (~3 g) were inoculated into 50 ml thioglycolate broth, and meconium specimens taken by a great loop (3 mm in diameter) were cultured in 5 ml thioglycolate broth. The cultures were incubated in the refrigerator at 4°C for several weeks. After 1, 2, 4, 8, 12, 20 and 26 weeks, samples from these cultures were smeared on nalidixic acid agar (40 µg/ml) and on nalidixic acid–trypaflavine agar plate (Trypaflavine-Hoechst, 45 µg/ml). The colonies were examined under oblique illumination [2] and those supposed to be *L. monocytogenes* were isolated on agar containing 5% sheep erythrocytes and identified by biochemical and serological methods and by means of animal experiments.

The samples of faeces originated from pregnant women who had agglutination titres 1 : 320 or higher (153 persons) and from mothers who had had a child with listeriosis (20 persons) and from healthy midwives (72 persons). In addition, 1170 samples of meconium were investigated in the period from May, 1971 till September, 1972. The results are demonstrated in Table I.

We succeeded in demonstrating *L. monocytogenes* on nalidixic acid–trypaflavine agar 10 times, whereas on nalidixic acid agar without trypaflavine only 5 times. Thus the nalidixic acid–trypaflavine agar proved to be a better medium. At the same time, *L. monocytogenes* could be isolated in four cases after a longer incubation period (10, 20, 33 and 49 weeks) also on nalidixic acid agar without trypaflavine. Four of the strains were of serotype 1/2a and six of serotype 4b. All strains were pathogenic for mice.

The number of colonies on nalidixic acid–trypaflavine agar was always higher than that on nalidixic acid agar without trypaflavine. The growth of accompanying flora (in most cases streptococci of the faecal group) was inhibited nearly entirely by trypaflavine at a concentration of 45 µg/ml.

Table I
Comparison of nalidixic acid-tryptose agar (NA) and nalidixic acid-trypaflavine tryptose agar (NATr)

No.	Name	Age, years	<i>Listeria</i> isolated after weeks' incubation	NA		NATr		Serotype	Origin of the sample	Observations
				No. of <i>Listeria</i> colonies	Contaminating germs	No. of <i>Listeria</i> colonies	Contaminating germs			
1	G. F.	42	26	0*	+	1	(±)	4b	midwife	—
2	H. S.	41	20	2	++	18	(±)	1/2a	mother	premature child, bacteriological listeriosis, alive
3	G. F.	42	12	0**	+	2	(±)	1/2a	midwife	—
4	R. P.	30	8	0***	+	16	(±)	4b	pregnant	titre, and H 1 : 320
5	Ch. M.	25	8	12	+	35	—	4b	pregnant	titre, and H 1 : 320
6	M. K.	24	1	0****	++	1	(±)	4b	mother	premature child died of listeriosis
7	D. F.	30 days	8	2	++	15	(±)	4b	infant	clinical and bacteriological listeriosis, alive
8	M. K.	24	12	4	++	10	(±)	4b	mother	premature child died of listeriosis
9	K. V.	28	2	0*****	++	6	(±)	1/2a	mother	child, clinical and bacteriological listeriosis, alive
10	K. G.	8 days	6	4	++	25	(±)	1/2a	premature child	healthy

— = no growth, (±) = strongly inhibited growth (only few colonies of faecal streptococci),

+ = moderate growth of contaminating germs, ++ = strong growth of contaminating germs

* = 1 colony *Listeria* isolated after 49 weeks of cold enrichment

** = 3 colonies *Listeria* isolated after 33 weeks of cold enrichment

*** = 4 colonies *Listeria* isolated after 10 weeks of cold enrichment

**** = 2 colonies *Listeria* isolated after 20 weeks of cold enrichment

***** = *Listeria* not isolated after 12 weeks of cold enrichment

Then 1170 samples of meconium were examined on nalidixic acid agar and on nalidixic acid-trypaflavine agar. We isolated *L. monocytogenes* in pure culture in 4 cases on both selective media. Thus, for this purpose, both media proved of equal value. The only difference concerning the two media consisted in finding 2 resp. 4 *L. monocytogenes* colonies on nalidixic acid agar, whereas 25 resp. 30 on nalidixic acid-trypaflavine agar (see Table I; Nos 7 and 10). These 2 samples were strongly contaminated by faecal streptococci.

We have intentionally reduced the concentration of trypaflavine in agreement with RALOVICH *et al.* [5], because it has become evident that in this basal medium the optimal growth of *L. monocytogenes* is inhibited by trypaflavine at a concentration of 50 µg/ml and the typical size, shape and colour of the colonies cannot be observed clearly before 72 hrs incubation (48 hrs at 37°C and 24 hrs at room temperature). After addition of trypaflavine at 45 µg/ml to the basal medium, the colonies were well-developed after 48 hrs at 37°C and they showed the typical shape and colour.

In summary, nalidixic acid-trypaflavine agar yielded better and earlier results for isolating *L. monocytogenes* from samples of faeces and meconium contaminated by faecal streptococci than did the nalidixic acid agar without trypaflavine.

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METHODS TO ISOLATE LISTERIA MONOCYTOGENES FROM DIFFERENT MATERIALS

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We have been studying the question of listeriosis since 1964. One of the questions was to solve the problem of selective cultivation and isolation of *Listeria monocytogenes* from different materials such as faeces, lochia, etc., thus from samples containing also other bacteria [1]. Till now we have applied three methods. Two of them have been published [2–5]. On the basis of the results obtained with the first method we may state that the incubation of specimens at 4°C for one month in Holman's medium containing filtered blood and the examination in oblique light of subcultures on 5% serum agar with nalidixic acid offer promising chances to cultivate and isolate *L. monocytogenes*. Nevertheless the presence of various Gram-positive cocci, not inhibited by nalidixic acid and the presence of the resistant mutants of some Gram-negative microbes caused difficulties during isolation.

When the growth of *L. monocytogenes* on Klimmer's plate had become known [6], the possibility of a new selective medium arose. Knowing that the tryptaflavine in Klimmer's medium inhibits Gram-positive cocci, we decided to test the combined effect of nalidixic acid and tryptaflavine. The medium and procedures applied were the same as before with the only difference of addition of tryptaflavine to the serum agar with nalidixic acid used for subcultures. After pre-enrichment at 4°C the cultures were smeared on serum agar containing nalidixic acid and tryptaflavine (NSAT plate). The optimum concentration of the inhibiting materials was between 40 and 50 µg per ml. The optimum quantity of the two inhibitory substances had to be pretitrated for every basal medium. On such plates *L. monocytogenes* appeared usually in pure culture. Growth of other bacteria was infrequent and they appeared in an inhibited form as compared to *Listeria* colonies, which were discernible by the naked eye on the basis of their characteristic cultural morphology (Figs 1, 2).

After 1 or 2 days incubation *L. monocytogenes* yielded the largest colonies yellowish-greenish in colour, of smooth surface, flat and transparent if checked in oblique illumination. Under the microscope the moiré effect of their iridescent surface was easily perceptible and so the colonies were unmistakably

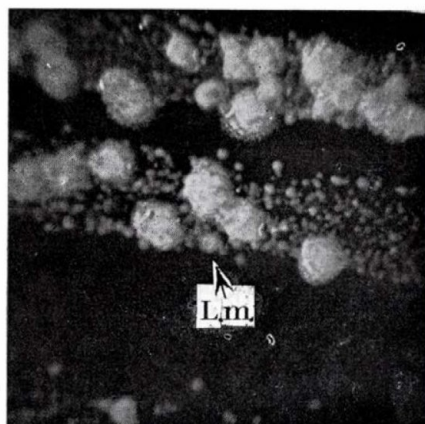


Fig. 1

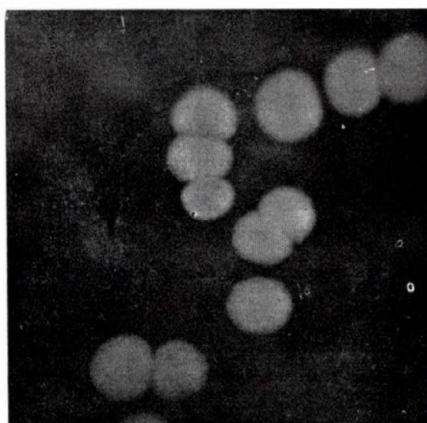


Fig. 2

recognized. A more transparent, greenish-blue edge located around the less transparent, yellowish-greenish centre was found characteristic.

In agreement with others [7—10] we think that the NSAT plate is an optimum medium for isolating *L. monocytogenes* strains from different enrichment fluids. However, both our methods have a significant disadvantage in that pre-enrichment takes several weeks. When we were informed that FÜZI and PILLIS [11] proposed the use of broth with 3% potassium rhodanate for the selective isolation of listeriae, we have made a trial with the method in spite of the fact that the Gram-positive cocci and the members of the genus *Bacillus*, as well as other microbes, could multiply in the said medium. The

advantage of the medium is the short incubation period; we assumed that the growth of these bacteria could be inhibited by the use of our selective NSAT medium. Thus, we made enrichment in broth containing 3% potassium rhodanate and thereafter transferred the growth onto NSAT plate. The results, however, were inadequate, as the rhodanate broth was not usable for enriching.

So the best method is to enrich in Levinthal's broth containing tryptaflavine and nalidixic acid or in Holman's medium with tryptaflavine and nalidixic acid, at 37°C for 2—4 days.

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DIFFERENTIAL MEDIA FOR THE ISOLATION OF *LISTERIA MONOCYTOGENES*

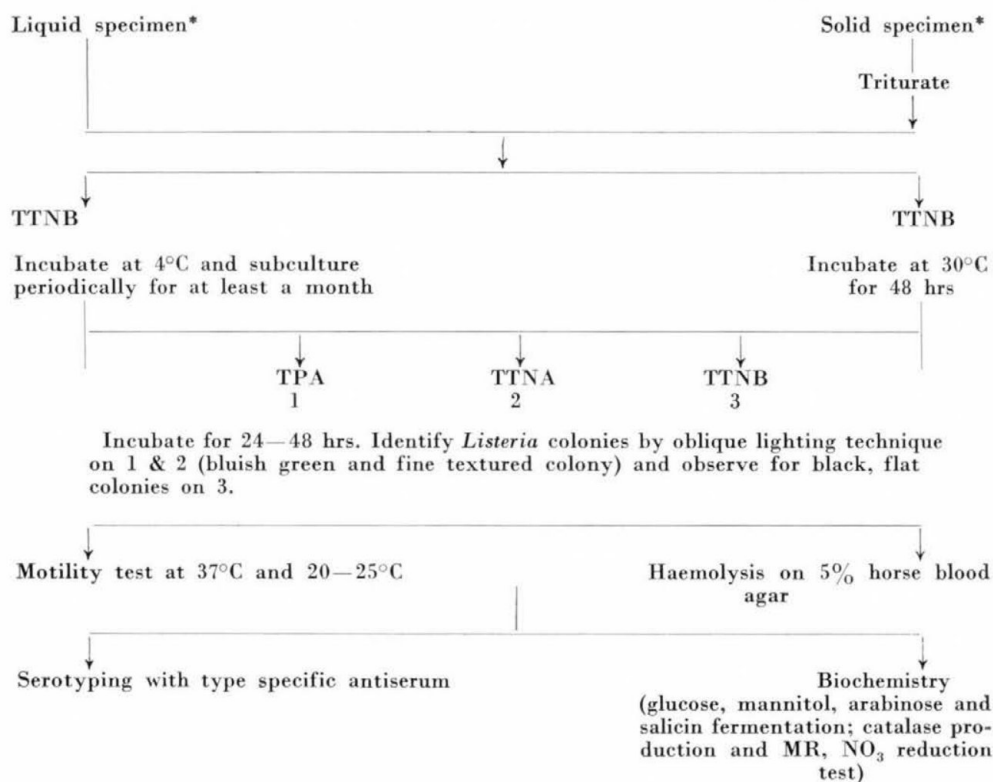
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A variety of selective media have been examined, including nalidixic acid; potassium tellurite; nalidixic acid; potassium thiocyanate; potassium tellurite; nalidixic acid potassium tellurite and thallos acetate potassium tellurite agars. A selective medium containing potassium tellurite (0.025%) and nalidixic acid (0.004%) with stock culture agar (Difco) as the basal medium was found satisfactory for the isolation of *Listeria monocytogenes*. On this medium 5 strains were isolated from 189 biological specimens and two from 14 silage samples. Growth inhibition studies, however, revealed that potassium tellurite (0.025%) was inhibitory to both fresh and old laboratory strains. No such inhibition of growth was observed with nalidixic acid (0.004%) alone and in combination with thallos acetate (0.02%).

A scheme for the isolation and identification of the bacterium involving different selective media and employing Gray's cold incubation technique is described (Table I). The previous finding that listeriosis of sheep may be caused by poor quality silage has been confirmed.

Table I

Scheme for the isolation and identification of *L. monocytogenes*

TTNB = Tryptose phosphate broth + 0.02% thallous acetate + 0.004% nalidixic acid.

TTNA = Same as above + 1.2% plain agar.

NPTA = Stock culture agar + 4 g/litre plain agar + 0.004% nalidixic acid + 0.025% potassium tellurite (add separately after autoclaving).

* If the specimen is not highly contaminated it may be streaked directly onto one of the selective plating media.

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IMMUNOFLUORESCENT DIAGNOSIS OF LISTERIOSIS; PREPARATION AND EXAMINATION OF CONJUGATED ANTILISTERIA SERA

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Immunofluorescent staining of *Listeria monocytogenes* was introduced by SMITH *et al.* in 1959 [1, 2] and then applied in the serologic analysis and diagnosis of this bacterial species. The data on typing of *Listeria* strains [1—11], cross staining of other bacterial species [1, 3, 4, 6, 7, 9], the identification of *L. monocytogenes* in preparations from experimentally infected animals [1, 2, 3, 5, 6] or patients [4, 5, 8, 12—15] do not seem to be adequate for its wider application in practice [16].

This study was aimed at preparing suitable labelled antilisteria sera for diagnostic purposes. The results are presented below.

The following *Listeria* strains were used: serotypes 1/2a-7973, 1/2c-5348, 3a-5105, 4a-5214, 4b-1071/53, 4c, 4d, 4e, 5 (Ivanov) and *Listeria grayi*. All the strains were obtained from Dr. H. P. R. SEELIGER. Strains from other bacterial species were obtained from the corresponding reference laboratories of RIEM (Sofia). The strains were maintained on blood agar; they were all S strains.

Considering that the antisera to types 1/2a and 4b are most widely used, sera were prepared against these serotypes. Bacterial suspensions containing 500×10^6 organisms per ml were prepared according to SEELIGER [17] and used for immunization. Groups of 6 chinchilla rabbits were immunized intravenously with increasing doses of the suspensions (0.5; 1; 1.5; 2; 2.5; 3 ml) at intervals of 4—5 days. The animals were bled 8 days following the last injection. Two sera of each type with the highest agglutinin titre were labelled with fluorescein isothiocyanate (FITC).

The whole gamma globulin fraction was fluorescein labelled (20 mg fluorochrome per 100 mg protein). The antiserum to 1/2a had a MF/MP ratio of 3.8, whereas the ratio for 4b antiserum was 6.8. Both sera were used at a dilution of 1 : 16, which gave 3—4+ fluorescence with the homologous bacterial suspensions.

The properties of the sera were examined by: (1) staining of preparations from the standard listeria strains enumerated; (2) staining of preparations from the following bacterial species: *Corynebacterium diphtheriae*, *C. hoffmanni*,

C. xerosis, *Erysipelothrix rhusiopathiae*, *Bacillus subtilis*, *Streptococcus* group A and D, *Staphylococcus albus*, *Staph. aureus*, *Escherichia coli*; (3) staining of slide prints from organs of albino mice, infected with listeriae.

The bacterial preparations were made from 24-hr glucose agar cultures suspended in physiological saline and killed by the addition of 0.5% formaldehyde. The preparations were fixed by heat.

The albino mice which had been inoculated intraperitoneally, except those inoculated with strains 5105 and *L. grayi*, died and were examined 96 hrs after the inoculation. The mice, inoculated with *L. grayi* and strain 5105, were killed and also examined 96 hrs later. Slide prints were prepared from liver and spleen, the former were fixed in acetone at 4°C for 30 min. The preparations from lung and brain were discarded because the results were unsatisfactory.

All the preparations were stained at room temperature in a humid chamber for 30 min. The slide prints were stained negatively with Evans Blue 1 : 2000, and washed in PBS, pH 7.4, for 10 min.

The preparations were examined under an Ortholux microscope, provided with an objective $\times 90$ and oculars $\times 6.3$. A Bg12 excitation filter and a K510 protection filter were used.

Table I illustrates the degree of fluorescence after staining preparations made from different reference *L. monocytogenes* strains. The agglutinin titres of both sera prior to labelling are also shown.

Table I

Agglutination and immunofluorescence of L. monocytogenes serotypes with antiserum 1/2a and antiserum 4b

Strains	Agglutinin titre		Degree of fluorescence	
	1/2a	4b	1/2a	4b
7973	1 : 3200	1 : 400	++/+++	±
5348	1 : 1600	0	++	±
5105	1 : 800	1 : 400	++	±
5214	0	1 : 400	±	±
1071/53	1 : 200	1 : 3200	±	++/+++
Type 4c	0	1 : 1600	±	++/+++
Type 4d	0	1 : 1600	±	+++ /++++
Type 4e	0	1 : 1600	+	++/+++
Type 5	0	1 : 1600	±	++
<i>L. grayi</i>	0	1 : 200	0	0

Table II

Immunofluorescence of slide prints of organs of albino mice infected with L. monocytogenes

Strains	Bacteriological examination	Ordinary staining with serum				Negative staining with serum			
		1/2a		4b		1/2a		4b	
		spleen	liver	spleen	liver	spleen	liver	spleen	liver
7973	positive	++	0	±	0	0	++	0	0
5348	positive	+++	+/++	0	0	+/++	++	0	0
5105	negative	0	0	0	0	0	0	0	0
5214	positive	0	±	0	±	0	0	0	±
1071/53	positive	0	0	++/++++	++/++++	0	0	++/++++	++/++++
Type 4c	positive	0	0	++/++++	++	±	0	++	++
Type 4d	positive	0	±	+++	++	0	±	++	++
Type 4e	positive	±	0	++	+/++	0	0	++	+/++
Type 5	positive	0	0	+/++	+/++	0	0	+	+/++
<i>L. grayi</i>	negative	0	0	0	0	0	0	0	0

The preparations from other bacterial species did not stain or stained slightly. Only one strain of *C. xerosis* and one of *Staph. aureus* showed a + to ++ fluorescence.

Results of the fluorescence-microscopic examination of slide preparations from albino mice are presented in Table II. Though several mice were infected with *L. monocytogenes* serotype 3a strain 5105 and *L. grayi*, the bacteria could not be demonstrated in the internal organs either bacteriologically or by immunofluorescence.

Pure bacterial cultures manifested typical peripheral fluorescence as shown by other workers. Some of the cultures, though S forms, contained cells emitting fluorescence of various intensity. This may be accounted for by S → R transitions or +/— variations.

In slide preparations from internal organs, in addition to the fluorescing bacterial cells, the cellular elements showed considerable fluorescence. Their accumulation was a serious obstacle in counting the fluorescent cells and reading their intensity (the number of fluorescent cells ranging from 3—4 to 20—30 in positive preparations).

Both sera possessed good staining properties and nearly all the representatives of *L. monocytogenes* could be differentiated. *L. grayi* was stained by neither of the sera, while strain 5214 of serotype 4a was stained faintly. This was the case with other strains belonging to 4a [4, 11], probably due to factor 5, which is not always found in serotype 4a. The irregular staining of the representatives of serotype 4a by anti-serotype 4b serum was not an obstacle in diagnosis, since it is a rare finding in humans. However, when preparing antisera against the second serogroup, serotypes containing antigenic factors common with serotype 4a should also be included.

Most of the workers [1, 3, 4, 6, 7, 9] point out that antilisteria sera do not stain other organisms except certain staphylococci. Our studies have confirmed these findings. The difference in the degree of fluorescence and the morphology of the microorganisms allows the differentiation between *L. monocytogenes* and other microbial species, especially staphylococci.

Negative staining of the slide prints with Evans Blue did not yield better results than ordinary staining; this was in agreement with the concept of BIEGELEISEN [4] and VILLELLA et al. [15] that negative staining is not necessary.

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LISTERIOSIS IN PROFESSIONALLY EXPOSED PERSONS

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Knowledge regarding the epidemiology of listeriosis has become deeper in recent years. Records of the recovery of *Listeria monocytogenes* from the faeces of healthy persons appear to confirm the existence of listeria carriership as the potential reservoir of human infection [7, 11, 14]. Listeriae were found in soil, plants, water and on the surface of various objects, and it was also found that listeriae can multiply and survive in outer environment for a long time [4, 9, 13, 16, 17]. As it is rare to find an animal source of infection in the close environment of sick persons, some authors ever cast doubt on the anthroponotic character of listeriosis [2].

In this paper we present a further study of the mechanism of *L. monocytogenes* transmission. In a district, 652 persons from various productive branches were investigated. Attention was focussed mainly on individuals professionally exposed to infection by contact with ill animals and/or animal products. Serological investigation was done by means of O and H agglutination and complement-fixation tests using antigens from *L. monocytogenes* serotypes 1 and 4. These antigens were prepared from the mixture of 6 strains according to the procedure of SEELIGER [15]. Two of these strains were type-strains, other four strains were isolated from animals in the region studied. Sera with a high titre of listeria agglutinins were tested also with staphylococci and enterococcal antigens and O and H agglutinins were differentiated into the IgG and IgM type by means of 2-mercaptoethanol treatment and inactivation at 65°C [12]. Staphylococcal antigen was prepared from strains isolated from persons involved in this study. Enterococcal antigen was prepared from *Streptococcus faecalis* serotype 10, 16 and 32 type-strains which were giving a distinct cross-reaction with listeriae [6].

Cultivation of listeriae from faeces and from swabs taken from the throat, nose, hands and gowns, was done in selected groups. Besides aerobic and microaerophilic cultivation, the cold technique in VF-broth at 4°C was used [5]. A number of faeces was treated by the dilution method and/or by 2-day incubation in KSCN-broth at room temperature [10, 16]. Cold incubation was later prolonged from 3 to 6 months using nalidixic acid agar with

sheep blood [1]. Swabs taken from the surface of hands and gowns were streaked on blood agar and blood nalidixic acid agar, incubated at room temperature in KSCN broth, and at low temperature in Vf-broth.

Fig. 1 shows results concerning O agglutination in four groups of persons, three of them professionally exposed. Group A consists of employees from two meat industrial plants, group B of employees from five farms where, approximately a month before this investigation, listeriosis in animals had been proven. In four of these cases *L. monocytogenes* serotype 1 and in one

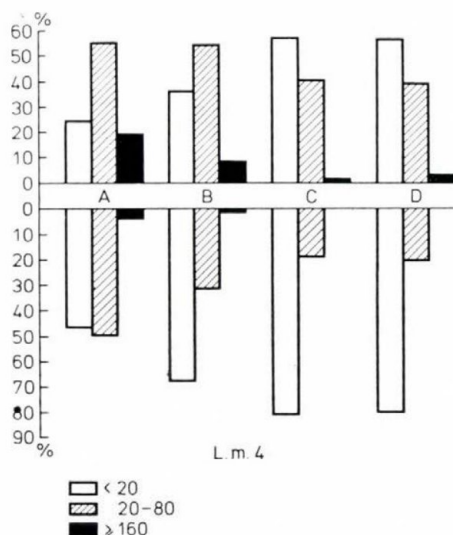


Fig. 1. Percental distribution of 652 persons according to *Listeria* O agglutinin titre: A — 245 workers in meat-industry; B — 103 workers in animal breeding; C — 101 workers in plant production; D — 203 persons, control group

case serotype 4 were found to cause the death of animals. Antibodies against *L. monocytogenes* serotype 1 were prevalent in persons from the investigated area when compared with antibodies against *L. monocytogenes* serotype 4 and this was in correlation with the distribution in animals of the mentioned serotypes. Animal listeriosis occurred mostly in the spring, when workers in agricultural centres and from the meat-industry were also investigated. We found significantly higher O agglutinin titres against *L. monocytogenes* serotype 1 in sera of employees from meat-industry as compared not only with a control group but also with exposed farm-workers from animal and plant production.

Comparing the O agglutinin titres against *L. monocytogenes* serotype 1 and *L. monocytogenes* serotype 4 in employees from various sections of the meat

industry, high titres were surprisingly frequent in workers employed in icehouse and in meat-production (e.g. sausage-production) (Fig. 2). Positive sera were tested simultaneously with enterococcal and staphylococcal antigens. Agglutinins were not found in dilutions higher than 1 : 80, and more than 50% of the examined persons were negative at the lowest dilution of 1 : 20.

We have also observed higher H agglutinin titres in sera of workers from the section of meat-production than from the other sections, though the differences were not as significant as for O agglutinins. H agglutinins against type 1 antigen were demonstrated in 6.9% of the workers from the meat-

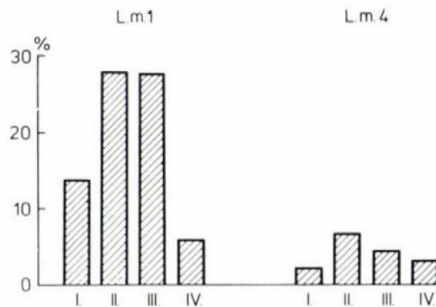


Fig. 2. Percentual occurrence of high titres (> 160) of *Listeria* O agglutinins in workers from different branches of meat-industry: I. 95 persons — slaughter-house; II. 47 persons — section of meat production; III. 69 persons — ice-house; IV. 34 persons — section of transport

industry and in 4.8% of those from animal breeding. Among the employees of the meat-industry higher titres of H agglutinins against *L. monocytogenes* serotype 1 were found in workers from the section of meat-production (12.8%). In this group, H agglutinins for *L. monocytogenes* 1 at dilutions of 1 : 40—1 : 80 were demonstrated in 55.8%, while workers from other sections were positive at these serum dilutions only in 23—31%.

Fig. 3 shows results for workers from the meat-industry, agricultural centres and for a control group tested by complement fixation. At initial serum dilution 1 : 4, 52.5% of the workers from the meat-industry were positive with type 1 antigen. The positivity of the complement-fixation test was significantly higher in this group of workers than in the other groups. The positivity of the complement-fixation test in the group of persons in contact with meat was in agreement with the fact that O and H agglutinins resistant to 2-mercaptoethanol and to heat are often detected in such persons. After

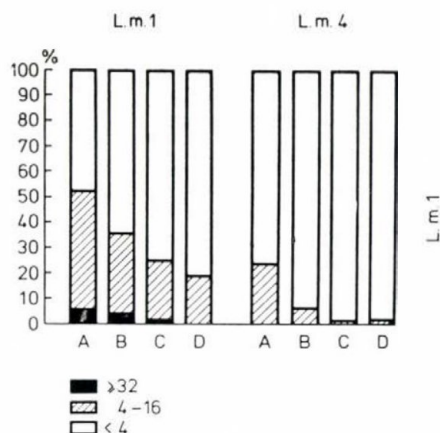


Fig. 3. Percentual distribution of 529 persons according to *Listeria* CF antibody titre: A — 198 workers in meat-industry; B — 78 workers in animal breeding; C — 73 workers in plant industry; D — 180 persons, control group

2-mercaptoethanol treatment and heat inactivation, O agglutinins persisted in 18.9% and H agglutinins in 50% of the serum of 37 employees of the meat-industry.

The results of bacteriological investigations are summarized in Table I. For technical reasons it was impossible to investigate all professionally exposed employees, therefore we have focussed attention on the employees of the meat-industry who had high antibody titres in previous tests. Of the 13 isolated strains 11 belonged to serotype 1 and two were nonhaemolytic avirulent variants of serotype 4. The latter two had been isolated from persons work-

Table I

Results of bacteriological investigations on listeriosis in professionally exposed persons

Material	Groups of investigated employees					
	Meat industry		Animal breeding		Plant production	
	Investigated	Positive	Investigated	Positive	Investigated	Positive
Throat swabs	49	0	98	0	62	1
Nasal swabs	49	0	98	1	62	0
Faeces	49	7	98	1	57	0
Swabs from hands	30	1	—*	—	—	—
Swabs from gowns	30	2	—	—	—	—

* not investigated

ing in different sections of the meat-industry, one from faeces, the other from the surface of a gown. Only one strain was isolated after incubation in KSCN-broth at room temperature for 2 days. After 4 weeks of cold incubation, 3 strains were isolated, after 4 months 1 strain, and 6 strains were isolated after 6 months of cold incubation. On prolonged cold incubation, the number of the isolations increased, however, not from the group of farm-workers.

All persons giving a positive cultivation result were clinically healthy, their personal and family history showed, however, some suspicious data. *L. monocytogenes* was positive more often in faecal specimens of workers from the section of meat-production and ice-house (6 strains) than in faecal specimens of workers from the slaughter-house (1 strain). This might have been due to the consumption of crude and semi-crude products during work.

As to pathologically terminating pregnancies of women working in the meat-industry, of 49 pregnancies in 31 women 11 had ended pathologically (dead foetus, spontaneous abortion, infants born before term). Especially premature labour was frequent (13%) in this group of women in comparison with the district average (1.2%) during the past three years.

Summing up, the most exposed group consists of workers in the section of meat-production and in the ice-house. In these cases the infecting agent enters the macroorganisms via the alimentary tract. One has to consider ingestion as a factor of infection in persons who do not come into contact with living animals but are working in a kitchen with meat potentially contaminated with listeriae, or consume semi-raw or raw meat products. The presence of *L. monocytogenes* in meat has been proven by others [3, 8]. The occurrence of clinically manifest listeriosis confirmed by cultivation in exposed persons is rare, and can be explained by the low virulence of listeriae and also by the technical difficulties of its routine bacteriological examination.

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SCREENING EXAMINATION FOR LISTERIOSIS IN PREGNANCY

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In the last 15–20 years listeriosis research has developed intensively. As listeria infection is often associated with abortion, premature birth or perinatal death, the problem bears a gynaecological and obstetrical importance.

Pregnancy and infancy present a special disposition: the data of OTTO [9] indicate that 31.8% of serologically and clinically diagnosed listerioses occurred in these groups of patients. SEELIGER *et al.* [15] reported that in the German Federal Republic in the period between 1950 and 1966, 58.1% of the infections affected pregnant women and infants. During the same period in the German Democratic Republic this value was 81.1%. In Hungary, DÖMÖTÖRI *et al.* [4], PATAKY and KEMENES [10], FAZEKAS *et al.* [5] and CZEIZEL *et al.* [3] reported on examinations in maternity and newborn wards.

Serological examination of pregnant in defined areas was performed by PATAKY and KEMENES [10], BODNÁR *et al.* [1, 2] and FAZEKAS *et al.* [6]. In Hungary, FAZEKAS *et al.* [7] were the first to isolate *Listeria* in connection with pregnancy and birth.

As serological tests are useful mainly for orientation, the need for extensive cultural examinations has recently been stressed [11, 15]. Cultural examination is important also in view of the fact that the clinical symptoms of listeriosis in pregnancy are not characteristic and symptomless listeria carriers are frequent.

Since these data showed the responsibility of *Listeria* in 1–7% of perinatal mortality [8, 13, 16] we have made screening examinations among pregnant in the municipal area of Kecskemét.

The survey was started in November, 1970. A total of 978 persons were examined. Vaginal and faecal specimens were cultured as described by RALOVICH *et al.* [12].

Listeria was cultured from 19 persons; 12 showed symptoms suggestive of listeriosis, 5 had influenza-like complaints; in the early stage of pregnancy 3 of the patients had abdominal and lumbar pain and endometritis with blood-stained discharge, 4 patients suffered from mild cystopyelitis.

Table I
Course of pregnancy in L. monocytogenes positive women

Patient	Previous pregnancy				Present pregnancy		
	abortion		birth		birth	month	therapy
	spontaneous	artificial	full-term	premature			
L. F.	—	—	1	—	term	8th	S + T
P. B.	—	2	4	—	premature	—	—
L. I.	—	1	—	2	premature	6th	S + T
K. J.	—	—	—	—	term	8th	S + T
N. P. K.	—	—	—	—	term	3rd + 7th	S + T + Ampicillin
D. S.	—	—	—	—	term	4th	S + T
K. A.	—	—	—	—	term	8th	T
M. S.	—	—	—	—	term	7th + 8th	T
B. S.	—	—	—	—	term	7th	Erythromycin
T. J.	—	—	—	—	term	4th	T
B. Z.	—	—	—	—	premature	7th	S + T
K. L.	—	—	—	—	term	7th	S + T
Sz. A.	3	—	—	—	term	4th	S + T
S. L.	—	—	1	—	term	5th	S + T
Sz. I.	—	1	—	—	term	5th	T
P. Gy.	—	1	1	—	—	7th	T
A. J.	1	—	—	—	—	5th	S + T
B. L.	—	—	—	—	—	—	—
V. I.	—	3	1	—	—	—	—

S = sulphamethoxypyridazine
T = oxytetracycline

Prophylactic treatment was prescribed between the 4th and 8th month of pregnancy for 16 out of the 19 patients. Serial bacteriological examination was made in 4 patients, of whom 3 had had sulphamethoxypyridazine and oxytetracycline treatment. All patients had become negative for *Listeria* by the end of pregnancy. One patient, receiving ampicillin was negative on 4 consecutive examinations, but the 5th faecal specimen obtained soon after delivery was positive.

Two cases are worth mentioning in detail. One patient, who had had 3 spontaneous abortions, was shown to carry *Listeria* during her 4th pregnancy. After sulphamethoxypyridazine + oxytetracycline therapy she gave birth to a mature baby weighing 3350 g. This woman worked in the refrigerator store

of a firm selling game. The other patient had a history of a premature and a term delivery with both infants dying in a few days. Necropsy of the infants suggested listeriosis, but no bacteriological examination was performed. At the next pregnancy the patient was shown to carry *Listeria*. After treatment she gave birth to a healthy 6-month premature baby weighing 2180 g.

The present examinations showed that 2% of the pregnant women carried *Listeria*. If this ratio is valid for Bács-Kiskun county, on the basis of an estimated number of 7700 births and 2500 spontaneous abortions, the number of *Listeria* carriers may be about 200. Their incidence in Hungary, taking 200,000 births and spontaneous abortions into consideration, may be estimated at 4000.

In view of the present results it may be stated that the introduction of compulsory screening of pregnant women would result in a decrease of perinatal mortality.

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SEROLOGICAL SCREENING OF PREGNANT WOMEN FOR LISTERIOSIS

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The difficulties of bacteriological identification have been an obstacle to the clarification of the role of listeriosis in pathological pregnancy. As to the serological tests, their correct evaluation requires caution, an allowance for cross agglutination, the use of uniform antigenic preparations and a control group chosen adequately [6, 8, 10].

In our first studies the method of "directed serological screening" was used. In view of the high titres found in cases of abortion or a still-birth it seemed necessary to compare the serological results to the agglutination titres obtained from control individuals and from healthy pregnant women.

For this reason, listeria agglutination was carried out in blood samples taken from all pregnant women reporting for the first time in the area of our maternity care service in the period from May 1, 1968 to April 30, 1969. In the patients showing increased titres the serological tests were repeated 4—8 weeks later. In those exhibiting high titres, isolation of *Listeria monocytogenes* from faeces, urine, and vaginal, cervical, and throat secretion, was attempted. The serological tests were repeated in 662 women admitted for delivery or abortion 24 hrs after the obstetric event. Titres obtained for 60 healthy males by the County Blood Donor Centre and those for 143 workers of the County Meat Industry Enterprise (77 males and 66 females) were used as controls [3]. The serological results obtained for 115 women with spontaneous abortion during autumn, 1968 [2] and those for 126 women delivering new-borns dying perinatally in 1968, were also evaluated. The 1921 serum samples collected were tested with living listeriae by a method described earlier [4].

The agglutination results are shown in Fig. 1. Titres of 1 : 640 or higher were found in 8% of the males and in 26—33% of the pregnant and control women.

Comparing the serological results obtained for healthy pregnant women to those for women undergoing spontaneous abortions or giving birth to new-borns that died perinatally (Fig. 2), no significant difference was found in the

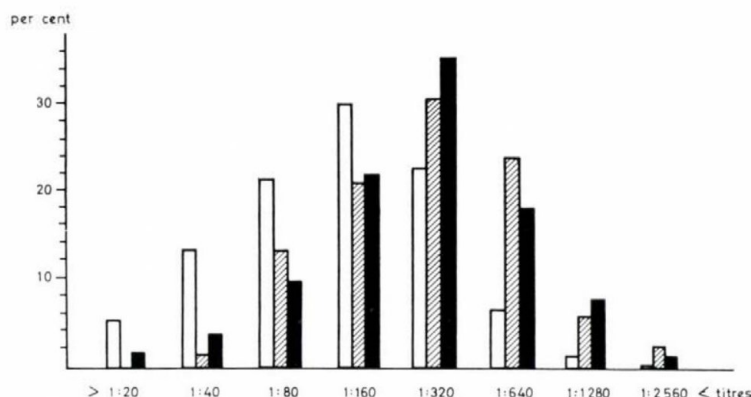


Fig. 1. *Listeria* agglutination titres in the sera of pregnant women and controls. □ males (137 cases); ▨ females (slaughter-house workers, 66 cases); ■ healthy pregnant (745 cases)

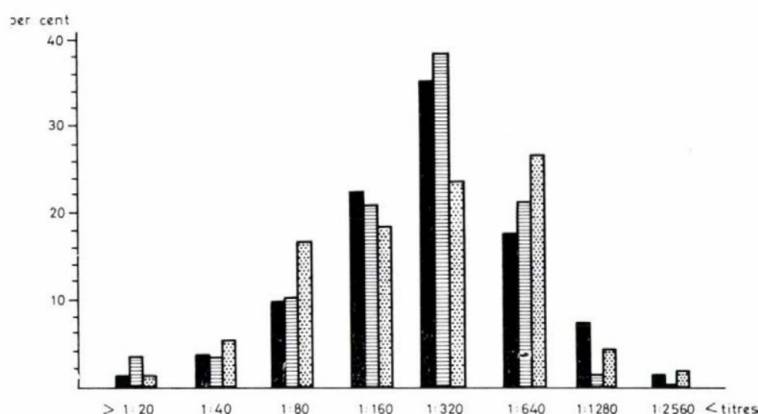


Fig. 2. *Listeria* agglutination titres in the sera of women with normal pregnancy, miscarriage, and still-birth. ■ healthy pregnant (745 cases); ▨ spontaneous abortions (115 cases); ▤ still-births (126 cases)

frequency of high titres between the healthy and the pathological groups ($\chi^2 = 0.44$ and 2.43, respectively).

Serological screening of pregnant women was made systematically over one year. The number of cases studied showed a monthly fluctuation between 47 and 83. The frequency of high titres exhibited a definite seasonality with a peak in the period between February and April (Fig. 3).

Distribution of the titres according to the occupation of the pregnant women (or of their husbands) showed little difference between those with agricultural and those with other occupations (Fig. 4). It was only the titres

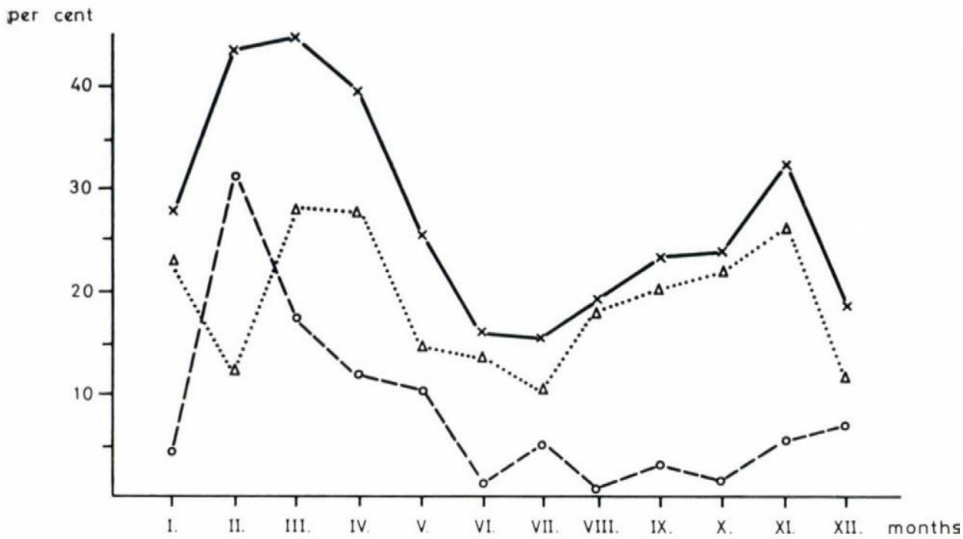


Fig. 3. Monthly changes in the frequency of listeria agglutination titres. $\Delta \cdots \cdots \Delta$ 1:640; $\circ \cdots \cdots \circ$ 1:1280 and higher; $\times \cdots \cdots \times$ total of titres above 1:640

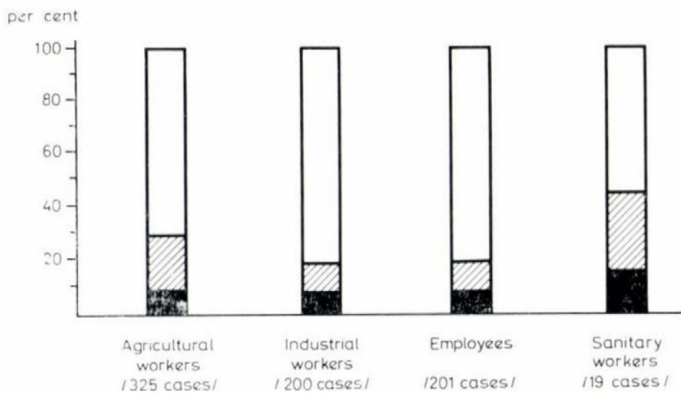


Fig. 4. Distribution of titres of pregnant women according to occupation. $\square$ 1:320 and lower; |||| 1:640; $\blacksquare$ 1:1280 and higher

obtained for the pregnant sanitary worker women that showed a more marked shift towards higher values. However, this finding must be assessed with caution in view of the small number of cases.

No significant difference in the titres was found when the results were grouped according to the pregnant's age or to the birth order. The result was similar when the titres were evaluated according to residence (urban or rural). These findings were in good agreement with the data of SEELIGER *et al.* [9] who

also failed to detect any significant difference between the titres of the urban and those of the rural population.

As indicated above, in patients with higher titres the serological tests were repeated once, or sometimes more than once, and bacteriological studies were performed. Though a significant increase or decrease in the titres could be observed in part of the women during pregnancy, trials to isolate listeriae were negative in all the cases.

The fate of 662 pregnant women was followed up. Of these, 3.5% ended with abortion, 8.3% with premature delivery while 88.2% with delivery at

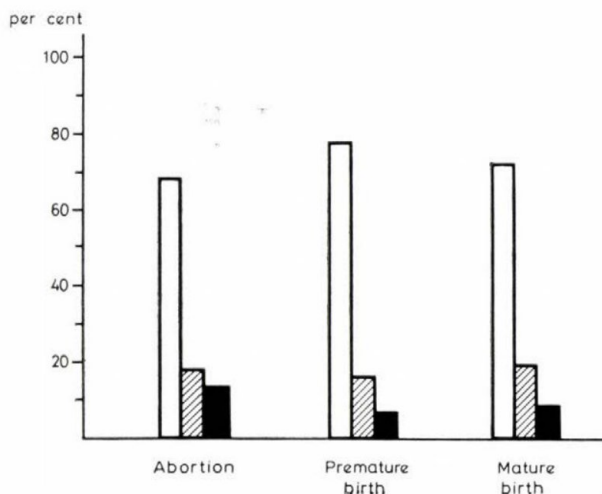


Fig. 5. Distribution of listeria agglutinin titres in pregnant women in relation to the obstetric event. □ 1 : 320 and lower; ▨ 1 : 640; ■ 1 : 1280 and higher

term. Distribution of titres was similar in the groups of term and premature births and a greater frequency of higher titres (1 : 1280 or higher) was observed only in women suffering abortions (Fig. 5).

In our earlier studies [1], high antibody levels against *Listeria* were frequently found in complicated pregnancies and in the mothers of foetuses died perinatally. The results for the 745 healthy pregnant women showed that the distribution of titres was practically the same in the normal pregnancies and births as in those with complications. No correlation between the outcome of the pregnancy and the titres showing an increasing or decreasing tendency during pregnancy, could be observed.

On the other hand, significant differences in the agglutination values for males and in those for pregnant or non-pregnant women were found, sup-

porting the data of RALOVICH *et al.* [7, 8] and of others [5]. As to the interpretation of the high values obtained for pregnant and non-pregnant women, one can only speculate.

Studying 800 patients with a suspicion of listeriosis, SEELIGER *et al.* [9] found that antibodies against O1 and O4 with a titre between 1 : 50 and 1 : 400 occurred in 75 and 45% of the cases, respectively, while H antibodies had a frequency of 10%. The frequent occurrence of O agglutinins raised doubts as to their specificity and the estimation of the H titre was considered as a possible means for serodiagnostics.

All those described above indicate that it is difficult at present to diagnose listeriosis merely by serological methods. Consequently, further investigations are needed to develop serological or other procedures which after proper standardization would offer a possibility for the detection of listeria infections and would be suitable for the demonstration of the presence (or specificity) of high diagnostic titres observed in pregnant women.

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LISTERIOSIS IN PUERPERAL WOMEN AND THEIR INFANTS

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Since the description of human listeria meningitis [4–6], extensive studies have revealed that the pathogen caused illness prevalently in pregnant women and newborn infants [10, 11, 13, 14, 18]. Several attempts have been made also in Hungary to confirm the role of *Listeria monocytogenes* in spontaneous abortion and pathological delivery. The small number of examinations and the different methods used made an evaluation of the results difficult [3, 7–9, 12, 19]. In Hungary listeriosis in pregnancy and neonatal age has been shown in recent years [1, 2, 10].

Our experiments were aimed to throw light upon contradictory data. Bacteriological examinations were carried out on 142 obstetric samples (placenta obtained from spontaneous or habitual abortion, vaginal discharge, lochia). The results have been presented elsewhere [15].

Having been unsuccessful in isolating *L. monocytogenes* from these cases, we supposed that listeriosis failed to play an important role in spontaneous abortion and premature delivery [16].

In 1969, we improved our cultural method by applying a new culture medium which contained tryptaflavine and nalidixic acid [17].

The purpose of our new studies was to determine the occurrence of *L. monocytogenes* in the faeces of healthy puerperal women. Since February, 1971, bacteriological examinations of the faeces of healthy mothers and their new-

Table I
*Occurrence of Listeria monocytogenes in the faeces
of puerperal women*

Residence	Number of samples	Number of <i>L. monocytogenes</i> strains	Occurrence, per cent
Town (Pécs)	42	1	2.4
Country	111	6	5.4
	153	7	4.6

born infants have also been made. We were also interested in the antibody titre of the sera of mothers and of the cord blood of their infants against type 1/2a and 4ab *Listeria* strains (Table I).

As shown in Table I in 153 puerperal women the first faeces after delivery was subjected to culturing. Forty-two of the women were town-dwellers and 111 villagers. Women living in the outskirts of Pécs, where animal keeping could be supposed, and those being engaged in farm-work, were included in the second category. *L. monocytogenes* was isolated from the faeces of 7 mothers. Six strains belonged to type 1/2a and 1 to type 4ab. Six of the women lived in the country and one in Pécs. As suggested by several authors, the fact that women living in the country are more likely to be in contact with animals and to eat raw food, may explain the difference observed.

In 107 out of the above mentioned 153 mothers not only their own faeces but also meconium specimens of their newborn infants were subjected to bacteriological examination (Table II).

Table II
Occurrence of *Listeria monocytogenes* in mothers
and their newborn infants

Residence	Number of samples		Number of positive cases	
	mother (faeces)	newborn (meconium)	mother	newborn
Town (Pécs)	38	38	—	—
Country	69	69	2	1
	107	107	2	1

L. monocytogenes was isolated from the faeces of 2 from among the 107 mothers, while no pathogen could be recovered from the meconium of the infants of the two infected women. Then again *L. monocytogenes* was found in the meconium of an infant whose mother yielded a negative result. All these hosts were healthy.

The serum of 42 puerperal women and their infants were examined serologically. Boiled suspensions of type 1/2a and 4ab *Listeria* strains were used as antigen. The results are presented in Table III.

With 1/2a antigen no titre was found in two cases; in four cases the titre was higher than 1 : 320. The serum of 19 mothers failed to agglutinate 4ab type antigen and all titres were under 1 : 320.

In the newborn infants, antibodies against 1/2a antigen could be shown in 10 sera and against 4ab antigen in 5 sera. The titres obtained were low, even the highest failed to exceed 1 : 80.

Table III

Results of agglutination with type 1/2a and 4ab strain antigens

Type of antigen	Serum	Distribution of reciprocal agglutination titres							
		0	20	40	80	160	320	640	1280
1/2 a	mother	2	1	11	8	11	5	2	2
	newborn	32	2	5	3	—	—	—	—
4ab	mother	19	5	6	8	4	—	—	—
	newborn	37	—	4	1	—	—	—	—

No correlation was found between the titres of the mother and her infant. The titre was negative or low in the cord blood even when the mother's serum yielded a titre of 1 : 640 or higher. The agglutinin level was always lower in the cord blood than in the mother's serum.

The results suggested that healthy pregnant women may carry virulent *L. monocytogenes* and still deliver a healthy infant. This fact indicates that the pathogenicity of *L. monocytogenes* is relatively low for man and suggests that human disease develops only upon some effect upsetting the normal physiological conditions.

Titres under 1 : 320 do not seem to be of any diagnostic value. In the case of a titre higher than 1 : 80 in an infant's serum, the possibility of listeric infection must, however, be taken into consideration.

On the basis of their unfavourable experience in prevention, most authors recommend serological and bacteriological screening of every pregnant woman and a bacteriological examination of the infant's meconium, especially in suspect cases.

Though both listeria-carrying parturients and newborn infants were found in our material, pathological manifestations have not been observed. Despite this we share the view that the possibility of listeriosis must be considered and bacteriological and serological examinations are obviously necessary when pregnant develop fever or when influenza-like symptoms in the pregnant mother are associated with signs of an infection of the foetus and adequate treatment must be applied in the positive cases.

Appropriate prenatal care may reduce the occurrence of neonatal listeriosis, and may favourably influence perinatal morbidity and mortality.

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LISTERIOSIS AND GESTATION

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A total of 5375 sera of obstetric and gynaecologic patients were examined with the Indirect Haemagglutination Reaction (IHR) in our modification [1]. A positive reaction was obtained in 633 cases (12.1%). The patients were classified into 4 groups (Table I).

Table I
Results of IHR in 5375 women

Clinical groups	No. of women	IHR negative	IHR positive	positive per cent
1. Pregnant women with a successful obstetric history and primigravidae	2495	2310	185	7.4
2. Pregnant women with unsuccessful obstetric history (previous spontaneous abortion, still-born, dead children)	809	534	175	24.6
3. Women with imminent abortion	1446	1261	185	12.8
4. Women with toxæmia, nephropathy, sterility or information could not be obtained	726	608	118	16.2
Total	5375	4712	663	12.1

We have observed 317 women with negative toxoplasmosis tests. Thirty-one were Rh-negative. Fifty-five women are primigravidae. The other 231 had the following obstetric history.

Obstetric history of 231 IHR-positive women: previous pregnancy 727; artificial abortion 289; gestation 438 (normal delivery 98, premature delivery 15, still-born and dead children 45, spontaneous abortion 277, extrauterine pregnancy 3).

Of the 438 pregnancies, 325 (74.1%) ended with foetal or perinatal death. Among these, malformations were detected in 23 cases, heart anomaly in 2 cases (one living and one dead), mental defect in 5 cases (living), microcephaly in 1 case (living), pigeon-toe in 1 case (living), progressive muscular

dystrophy type Werdnig Hoffmann in 1 case (dead), absence of one kidney in 1 case (dead), hypoplasia of liver and spleen in 1 case (dead), hydrocephalus of newborn and children in 4 cases (dead), hydrocephalus of foetus in 3 cases (spontaneous abortion), apodia of foetus in 1 case (spontaneous abortion), short-legged foetus in 1 case (spontaneous abortion).

As Table II shows most spontaneous abortions (72%) occurred in the 16th week of pregnancy.

Table II
Distribution of 273 spontaneous abortions in the course of pregnancies

Weeks of pregnancy	0-11	12-16	17-22	23-27	28-30	Total
No. of abortions	132	65	31	27	18	273
Per cent	48.3	23.8	11.4	9.9	6.6	100

The 288 pregnant women showed the following clinical signs: elevation of temperature 85 (29.4%), influenza-like symptoms 81 (28%), painful urination 57 (19.7%), general weakness, rapid fatigue 114 (39.9%), pain in the uterine area 209 (72.5%), vaginal discharge 154 (53.4%), toxæmia of pregnancy 47 (16.3%), headache 48 (16.6%).

Sixteen women in the spontaneous abortion danger period were found to have a large and painful liver.

Haematologic examinations were made in two groups of women with imminent abortion, primigravida and multigravida up to 12 weeks pregnancy. In the first group were 31 women, all IHR positive for listeriosis. In the second (control) group were 29 women, all IHR negative. The results are given in Table III.

Table III
Blood counts in two groups of women with imminent abortion

	31 women IHR positive	29 women IHR negative	
Erythrocytes	3,470,000	3,740,000	/cu.mm
Haemoglobin	11.47	12.12	g per 100 ml
Sedimentation rate	12.0	17.6	mm/hr
Leucocytes	6100	7650	/cu.mm
Eosinophils	2.37	2.09	per cent
Band forms	11.70	9.83	per cent
Segmented neutrophils	54.74	58.20	per cent
Lymphocytes	25.58	24.10	per cent
Monocytes	6.35	6.00	per cent

The results obtained in these two groups revealed no significant difference. However, in the IHR positive women the SSR and the number of monocytes was normal, but the number of band forms was increased to 11.7%.

Of the material, 150 pregnant women were treated with antibiotics. The best results were obtained with a combination of tetracycline with penicillin. Tetracycline was given orally in doses of 25 mg/kg/day and parenteral penicillin in doses of 1 million IU four times daily. A course of treatment was continued for 7 days and it was repeated three times. Information could not be obtained for 29 pregnancies; 121 pregnant after treatment delivered healthy children, 105 pregnancies (107 newborns); newborn with congenital infection, 4 (5 newborns, 1 dead); congenital heart defect, 1 (living); caesarean section (healthy child), 1; stillborn, 2; spontaneous abortion, 8 pregnancies.

Thus, in the treated group, the frequency of an unsuccessful outcome of the pregnancy fell from 74.1% to 8.3%. In eleven children whose mothers had been treated with tetracycline during pregnancy, the deciduous teeth were yellowish in colour.

A total of 53 non-pregnant women with many unsuccessful previous pregnancies were treated with antibiotics in massive doses. During antibiotic treatment, 9 patients became pregnant; 8 of these patients gave birth to normal children, and one aborted spontaneously. After antibiotic treatment 16 women became pregnant and gave birth to healthy children, 8 had a history of delivering infants with malformations.

Three women with many spontaneous abortions in the history were unsuccessfully treated with repeated massive doses of antibiotics. They remained IHR positive and had spontaneous abortions.

The diagnosis of listeriosis must be based on the following data: IHR positivity; obstetric and gynaecologic history; clinical symptoms during pregnancy (weakness, periodical pain in the uterus area, vaginal discharge, toxæmia of pregnancy); clinical result of antibiotic treatment; the typical titre changes associated with the antibiotic treatment and the clinical condition of women; delivery of full term infant.

The clinical signs of listeriosis are not typical, but together with IHR positivity they indicate the necessity of prescribing antibiotics. The favourable effect of antibacterial therapy (disappearance of weakness and pain, absence of vaginal discharge, toxæmia and headache and an improved condition of the patient) means that the infection has been eliminated.

Our immunological results agree well with those of RABINOVITZ *et al.* [2].

The role of *Listeria monocytogenes* in human foetal losses and neonatal infection is clear from the data in Tables I and II.

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TETRACYCLINE TREATMENT OF PREGNANT WOMEN WITH SUSPECTED LISTERIOSIS

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In Bábolna, a village famous for its fowl, horse and swine breeding farms, the human birth rate and the incidence of mental deficiency were shown to be worse than the nation-wide average. Thus in 1966, 14 out of 31 newborn babies were under 3000 g in weight and 3 infants exhibited congenital deformity. Fifteen out of the 419 children of the village were attending school for the mentally defective.

In view of the above findings the role of *Listeria monocytogenes* was suspected. Serological examinations were performed in 176 pregnant women by using MÜLLER's method [1]. Titres 1 : 320 or above were considered to suggest the presence of listeriosis. The distribution of positive findings were: 40 patients, 1 : 320; 9 patients, 1 : 640; 1 patient, 1 : 1280. As recommended in the literature [2-4], these patients were treated with tetracycline, in a daily dose of 1 g. No side effects developed, only one patient had diarrhoea. Two patients were treated in the first, 45 in the second, and 3 in the third trimenon.

All 50 infants were observed for 4 years. The control group consisted of 50 infants of the same age whose mothers received no tetracycline. The results are shown in Table 1.

Mental and neural disorders were characterized by logopathy, primitive reactions, extreme shyness and vegetative disturbances. Constitutional disorders manifested themselves with a lower body weight, which may have been associated with the lower birth weight (5 out of the 7 newborn infants weighed less than 3000 g).

The following conclusions have been drawn.

1. Newborn babies of mothers receiving tetracycline weighed significantly more than those of untreated mothers.
2. Mental, neural and constitutional disorders were not observed in the children of treated mothers.
3. Dental coloration characteristic of tetracycline treatment appeared on the deciduous teeth of two children. Other complications such as deficient general or bone development, were not met with.

Table I

Observations on infants of mothers treated and not treated with tetracycline during pregnancy

	No. of infants from	
	treated group	untreated group
Birth weight above 3000 g	35	26
Birth weight under 3000 g	15	24
Mental affection	—	2
Nervous affection	—	2
Constitutional affection	—	7
Dental caries	2	2
Dental coloration	2	—
Strabismus	7	—

4. Seven children deriving from the treated group of mothers had strabismus, which was absent from 50 infants of untreated mothers. This finding might be associated with antibiotic therapy; it has been shown recently [4] that some infants treated with tetracycline develop abducent muscle paralysis. Accordingly, our observations support the opinion [2, 4, 5] that the use of ampicillin is more advisable.

It should be emphasized that the serological results pointed to not more than a suspicion of listeriosis, since culturing was positive in a single case only. Thus it is not proven that the favourable effect of tetracycline was due entirely to its effect upon *L. monocytogenes*.

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CONGENITAL LISTERIOSIS

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BURN [1] gives the first description of listeriosis in the newborn. The diagnosis of listeriosis requires bacteriologic and morphologic evidence. The paediatrician cannot wait for the exact bacteriologic diagnosis in case of such severe illness [2]. The object of the present investigation was to study newborn infections due to *Listeria monocytogenes* using the indirect haemagglutination reaction (IHR). Serum specimens were obtained from the maternity hospital. In the examination of 242 sera of parturients and puerperal patients a positive reaction was obtained in 39 cases. The results in the clinical groups are given in Table I.

Table I

Results of IHR for listeriosis of 242 pregnant and puerperal women

Clinical groups	No. of women	IHR- negative	IHR- positive	Per cent
1. Term birth or gestation of 36-40 weeks	119	108	11	9.2
2. Premature delivery	48	36	12	25.0
3. Imminent premature delivery	33	24	9	27.3
4. Still-born	11	7	4	36.3
5. Other diagnosis	31	28	3	9.6
Total	242	203	39	16.1

Out of 39 IHR-positive women 13 were primigravidae, the other 26 had the following obstetric history: previous pregnancy, 114; artificial abortion, 30; gestation, 84; normal delivery, 21; premature delivery, 6; still-born and dead child, 16; spontaneous abortion, 41.

Out of 84 pregnancies 57 ended with foetal death, still-birth or dead children.

All the children from these 39 pregnancies were delivered. Normal children, 12 pregnancies; newborn with congenital infection, 17 pregnancies; still-born and newborn dead, 10 pregnancies.

In addition, the nine pregnant women (six with premature delivery, two with nephropathy, one with toxicosis) were treated with penicillin and tetracycline in combination. The clinical results were good and the patients gave birth to normal infants in six cases and with congenital infection in three cases.

In the following the clinical picture observed in 12 cases out of 17 newborns with congenital infection will be described and the symptoms listed in Table II.

Table II
Connatal signs in 12 infants

Infants weights, g	Hepa- tome- galy	Sple- nome- galy	Conjunc- tivitis	Skin eruption	Pneu- monia	Hypo- tonic muscles	Are- flexia	Hyper- thermia	IHR-titre (mother)	Outcome
1. 2900	+	—	—	—	+	+	+	—	1 : 640	Recovery
2. 2500	+	+	—	—	+	+	+	—	1 : 80	Recovery
3. 3100	+	+	—	—	—	+	+	—	1 : 40	Chronic listeri- osis
4. 2800	+	—	—	+	+	+	+	—	1 : 40	Death
5. 3500	+	—	—	—	—	+	—	+	1 : 160	Chronic listeri- osis
6. 3850	+	—	—	+	—	+	+	—	1 : 5120	Recovery
7. 2600	+	+	—	—	+	+	+	—	1 : 640	Recovery
8. 2450	+	+	—	—	—	+	+	—	1 : 80	Recovery
9. 4150	—	—	+	—	—	—	—	—	1 : 160	Recovery
10. 3200	+	+	+	—	+	+	+	—	1 : 80	Recovery
11. 1600	—	—	—	—	—	+	+	—	1 : 80	Death
12. 1800	—	—	—	—	+	—	—	—	1 : 160	Recovery
Total	9	5	2	2	6	10	9	1		

L. monocytogenes was cultured from liver and lochia (Case 4), from meconium (Case 9) and from lochia (Case 11)

The leading pathognomonic symptom in congenital listeriosis of full term newborns is a solid and large liver. The connatal character of the clinical signs (hepatomegaly, splenomegaly, conjunctivitis, exanthem) and positive test (IHR) for listeriosis in the mother's serum is an essential condition for the clinical diagnosis of congenital listeriosis.

Case 1. Primigravida, 17 years, menstrual history non-contributory, last menses 22—23 November, 1965. 15 July, 1966: delivery, the amniotic fluid was turbid; girl 2900 g, 49 cm; not crying, cyanosis of lips, auricles, fingers;

muscular hypotonia, skin and mucous membrane reflexes are not marked, heart rate 100 per minute, and respiratory rate 90 per minute. Auscultation of the chest revealed moist rales and weak heart sounds. The liver reached to the level of the navel, it was solid and its edge blunt. The spleen was not palpable.

Laboratory. RBC, 4,900,000; haemoglobin 19 g per 100 ml; WBC, 9600, with 2.5% eosinophils, 7% bands, 78.5% segmented polymorphonuclears, 10% lymphocytes, 2% monocytes. IHR-positive 1 : 640 (mother). On the basis of the hepatomegaly and of the IHR-positivity with serum of the mother, congenital listeriosis was diagnosed. Therapy was started with cardias, oxygen, blood plasma, and parenteral penicillin, 350,000 IU four times daily. Symptomatic improvement was rapid. After three days therapy, the liver was just palpable. Penicillin treatment was continued for a total of 7 days. The girl was discharged in a satisfactory condition 11 days post partum.

Case 2. 25 years old, menstrual history non-contributory, married 3 years. Pregnancies: 1. 1965, artificial abortion. 2. 1965 gestation, last menses 7–10 July, 1965. She had toxæmia throughout the pregnancy and periodical pains in the area of the uterus in the sixth month.

On 23 March, 1966, she delivered a male infant, 2500 g, 45 cm, gravely asphyxiated. Auscultation of the chest revealed moist rales. Respiration was slow and shallow, heart sounds were weak, 155 per minute. The skin and mucous membrane reflexes were not marked. The liver was palpable 3 cm below the right costal margin, it was solid and the edge blunt. The spleen was not palpable.

Laboratory. RBC, 4,640,000, haemoglobin 20 g per 100 ml, sedimentation rate, 1 mm/hr, WBC, 15,200, eosinophils 3%, band forms 7%, segmented leucocytes 43%, lymphocytes 36%, monocytes 10%. IHR-positive 1 : 80 (mother). Therapy was started with cardias, parenteral penicillin and tetracycline, blood plasma, oxygen. Symptomatic improvement was rapid. Antibiotics were continued for a total of 7 days. The liver reached to the costal margin by the fifth day of therapy. The infant was discharged in a satisfactory condition at 25 days of age.

Case 3. 18 years old, virgin, menstrual history non-contributory. In August, 1965, she had had general weakness, rapid fatigue and pain in the area of heart. The clinical and laboratory findings were negative for rheumatism, brucellosis, syphilis, tularemia, toxoplasmosis, tuberculosis; the IHR for listeriosis was positive 1 : 40. Antibiotics were not given but her condition improved. In 1966, she married and became pregnant. During the 20th week of pregnancy she had become ill with general weakness, dull abdominal pain, uterine discharge. She was admitted to hospital with a diagnosis of imminent abortion. IHR-positive, 1 : 40. Treatment with tetracycline and penicillin for 7 days; the clinical result was good.

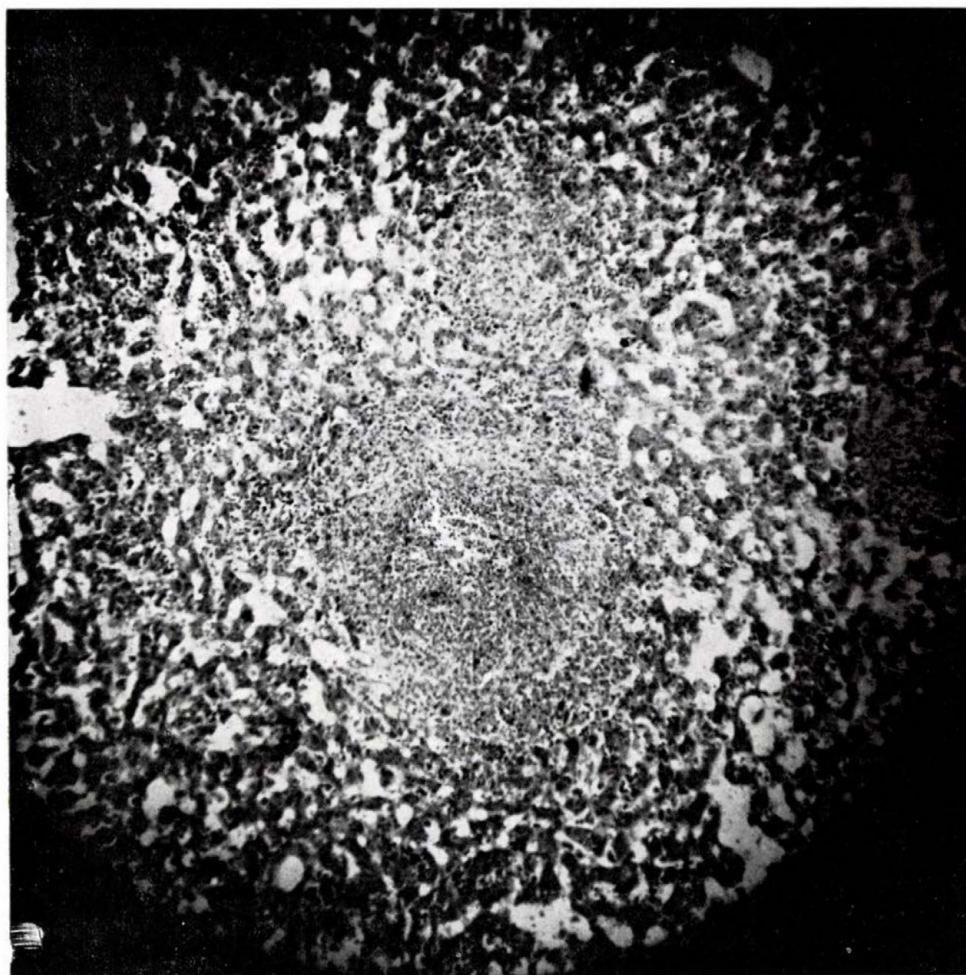


Fig. 1. Necrotic foci in the liver of a newborn infant 72 hours of age.
Magnification, objective $\times 9$; eyepiece $\times 15$

On 8 August, 1966, she delivered a male infant of 3100 g, 50 cm. Hypotonic muscles, the liver was palpable 2.5 cm below the right costal margin, it was solid and its edge was blunt. The spleen was palpable 1.5 cm below the costal margin. IHR-positive 1 : 40 (mother). Therapy: parenteral penicillin in a dose of 300,000 IU four times daily. Symptomatic improvement was rapid. The infant was discharged in a satisfactory condition at 14 days of age, with a weight of 3200 g. At 7 months of age the baby was depressed, flaccid, anorexic, the liver was enlarged to 3 cm below the costal margin. IHR-negative (baby). Treatment: antibiotics, intravenous plasma. Improvement. The liver was normal in size. At 19 months of age the baby was again depressed, flaccid,

anorexic, the liver reached 3.5 cm below the costal margin. IHR-positive 1 : 80 (baby). Diagnosis: chronic listeriosis. Treatment: tetracycline and penicillin for 7 days. The child became symptom-free after 5 days.

Case 4. A primipara of 24 years, menstrual history non-contributory. 1964, artificial abortion. 1966, artificial abortion. 1967, gestation. Last menses 10–13 June, 1967. During the 4th month of pregnancy she had had pyrexia and was admitted to a hospital, but the aetiology remained obscure. In the fifth month of pregnancy she had dull pains in the area of the uterus and toxæmia. On 1 April, 1968, she was admitted to a maternity hospital with a temperature of 38°C. There was leaking of amniotic fluid 62 hours before delivery. Treatment with penicillin in a dose of 15,000 IU six times daily, and streptomycin 0.250 g two times daily, sulphathiazole, 1.0 g four times daily. On 2 April she delivered a male infant of 2800 g, 49 cm. The infant was severely asphyxiated at birth, had a papular skin eruption, areflexia, hypotonic muscles. Auscultation revealed overall moist rales, heart sounds were weak, 160 per min. The liver was palpable 3 cm below the right costal margin. Treatment was initiated with cardiacs, oxygen, parenteral penicillin in a dose of 50,000 IU four times daily and intravenous blood plasma. The child died 72 hrs after birth.

Post mortem examination. The liver was enlarged to 3 cm below the costal margin and showed innumerable small (less than 1 mm) yellowish-white granulomas (Fig. 1).

The spleen was not enlarged. Sparse small granulomas were seen in the spleen, lungs, adrenal cortex, ileum. The left kidney was missing. The lungs were firm and contained oedema fluid. Microscopically, there were intra-alveolar erythrocytes and hyaline membrane. There was blood in the cerebral ventricles, the aqueduct and on the pia and dura.

Listeria monocytogenes were found in the liver and in the lochia. IHR-positive 1 : 40 (mother).

Pregnancy 4, 1968. Spontaneous abortion at 20 weeks. IHR-positive 1 : 80. Treatment with chloramphenicol, 100 mg/kg/day.

Pregnancy 5, 1969. IHR-negative. Last menses 20–23 November, 1969. The patient was healthy throughout pregnancy, and she gave birth to a healthy girl at term.

As the above cases showed, connatal hepatomegaly and IHR-positivity in the mother's serum are characteristic, and useful for rapid diagnosis.

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INVOLVEMENT OF THE CENTRAL NERVOUS SYSTEM IN NEONATAL LISTERIOSIS

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During the last 12 years we have observed 117 neonatal cases of listeriosis [1—8]. Two thirds of the patients were prematurely born babies, the rest mature infants (Fig. 1). The majority of infants were treated with oxytetracycline. Those cases which were treated with ampicillin have not been included in the above patient material.

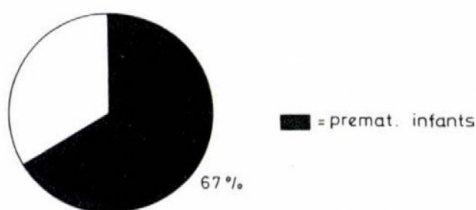


Fig. 1. Proportion of premature infants

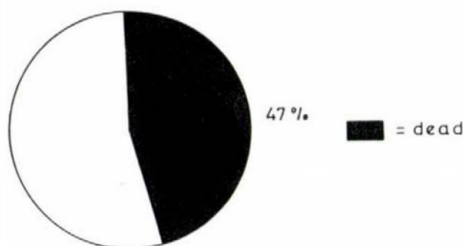


Fig. 2. Lethality

The lethality amounted to 47% (Fig. 2). The majority of patients died on the first days of life, supposedly before the therapeutical measures taken could become effective (Fig. 3).

So far as the clinical symptomatology is concerned, pulmonary phenomena were predominant. Central nervous symptoms were observed in 35% of the cases (Fig. 4), with seizures and somnolence followed by meningism and reflex anomalies. Palsies were observed in isolated cases only (Fig. 5). The

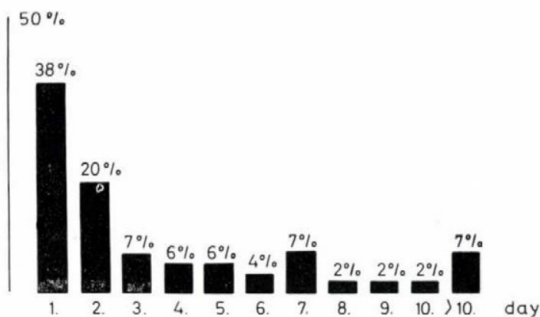


Fig. 3. Time of death

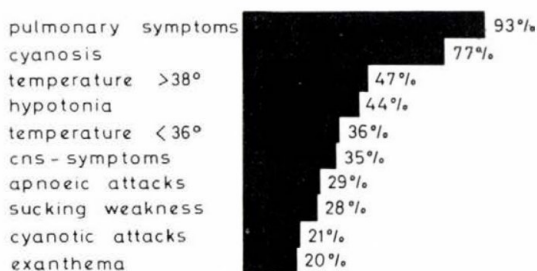


Fig. 4. Symptomatology

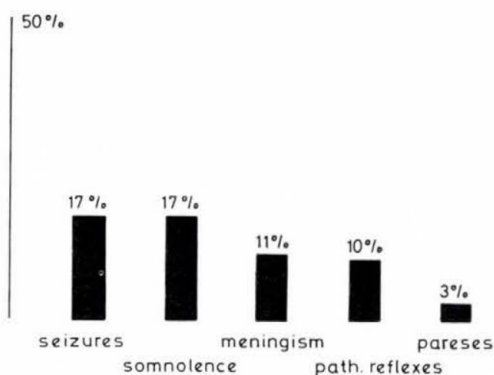


Fig. 5. CNS symptoms

meningitic signs were, in decreasing order, opisthotonos, tension of the fontanelles, sensitivity to touch, stiffness of the neck as well as positive Brudzinkin's and Kernig's signs. Spasms were considered a particularly unfavourable symptom inasmuch as 65% of these patients died, whereas among the patients displaying only other central nervous signs, lethality was far smaller (Fig. 6).

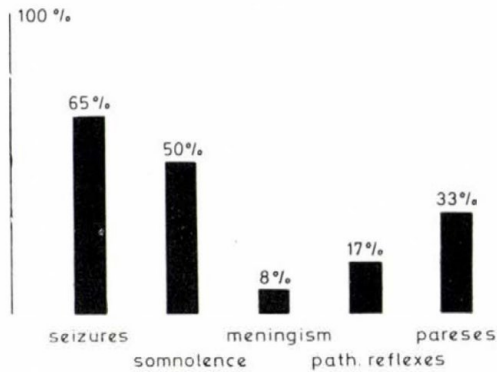


Fig. 6. CNS symptoms and lethality

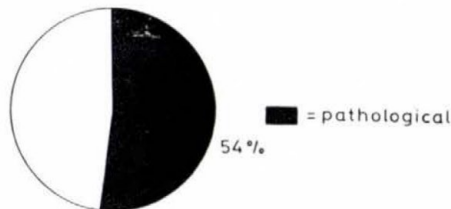


Fig. 7. Lumbar puncture

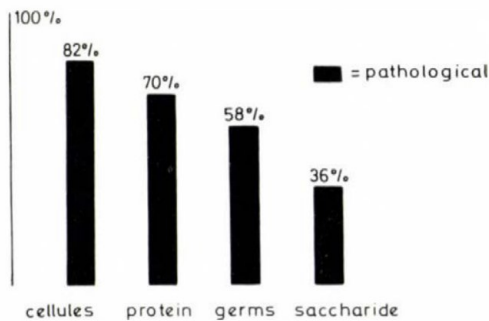


Fig. 8. CSF results

Lumbar puncture performed on 61 patients showed pathological conditions in 35 patients (Fig. 7). The CSF from 12 patients contained blood and had to be discarded. The other cases showed normal conditions.

The CSF of the 33 neonates showed pleocytosis in 27 cases, increase in protein in 23 cases, *Listeria* in 19 cases, and a decrease in the sugar content in 12 cases (Fig. 8). A number of patients showed normal cell counts in the CSF despite the presence of central nervous system listeriosis. In the others there was a pleocytosis of up to $10,000/3/\text{mm}^3$ and more, in some children the CSF protein content was above 1000 mg per 100 ml. The decrease of sugar

which in 7 patients was less than 20 mg per 100 ml with a minimum of 6 mg was a particularly unfavourable prognostic sign.

Listeria was detected in 82% of the patients examined, chiefly in meconium (63%) and nasal swabs (31%), less frequently in the CSF (20%) and in the pharynx and conjunctiva (9%) (Fig. 9).

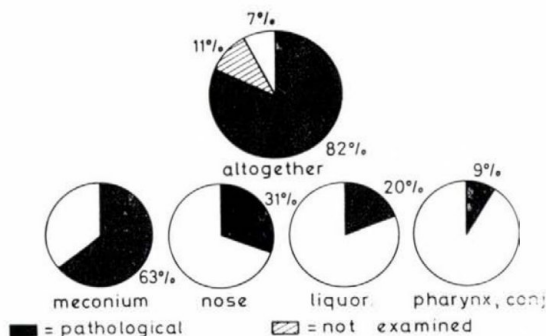


Fig. 9. Detection of listeria

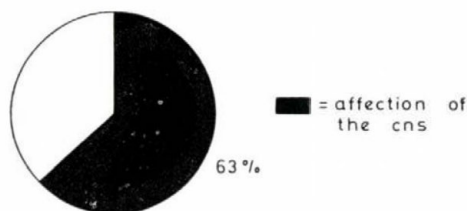


Fig. 10. Post mortem findings

Involvement of the central nervous system was found in 26 (63%) out of 41 cases with post mortem established listeriosis (Fig. 10). *Listeria* infection restricted to the CNS occurred in a single case; in the majority of cases, several organs were involved so that listeric meningitis developed within the framework of a septicaemia (Table I). A comparison of the affection of organs with and without CNS involvement showed that in the presence of meningitis more other organs were involved simultaneously (Table I). These were in the order of frequency, liver, lung, intestine, spleen, adrenals, oesophagus, kidney, pharynx, gastrointestinal tract, skin, middle ear, etc. (Table II).

A comparison of the clinical symptomatology with results for the CSF (Table III) showed that in all of the surviving neonates with CNS symptoms (19 cases) the CSF was pathological (punctures were dispensed with in 2 cases, while in 1 case the CSF contained blood). Also, lumbar puncture was dispensed with in 4 of the 22 deceased newborns with CNS symptoms as these babies were seriously ill when born or they died in the first hours of life. In 6 cases the

Table I
*Simultaneous affection of organs
 with and without listeria meningitis*

No. of organs	Listeria meningitis	
	yes	no
	No. of infants	No. of infants
1	1	3
2	1	4
3	1	1
4	—	4
5	4	2
6	6	1
7	5	—
8	3	—
9	1	—
10	2	—
12	1	—
13	1	—

Table II
Organs simultaneously affected in listeria meningitis

Organ	No.
Liver	23
Lung	22
Intestines	21
Spleen	18
Adrenals	18
Oesophagus	15
Kidney	7
Pharynx	5
Stomach, skin, middle ear	3 each
Palate, thymus	2 each
Tongue, trachea, pleura, gallbladder, urinary bladder, paranasal sinus, plica epiglottica	1 each
CNS only	1

CSF contained blood so that it was not examined further. As the results were pathological in all cases, CNS symptoms are a reliable indicator of listeria meningitis.

However, of the 22 deceased patients displaying clinical CNS symptoms in 4 cases the necropsy failed to reveal listeriosis either macroscopically or microscopically (Table III). Since 3 of these children showed pleocytosis in

Table III
Interrelation of findings

	Pathological	Normal	Not examined
I. CNS symptoms (n = 41)			
CSF (survivors)	16	—	3
CSF (deceased)	12	—	10
necropsy	18	4	—
II. Pathological CSF (n = 33)			
CNS symptoms	29	4	—
necropsy	7	3	—
III. Listeria meningitis at necropsy (n = 26)			
CSF	8	—	18
CNS symptoms	11	15	—

the CSF, there could be no doubt about the presence of meningitis; probably, regions of the brain other than those examined may have been affected. In the fourth case, the CSF contained blood, so that further examination was dispensed with.

The fact that in 4 out of all the patients with a pathological CSF no CNS symptoms whatever were observed is not uncommon since newborn infants with meningitis frequently show only general symptoms such as apathy, weakness, vomiting, etc., or may even be completely free of symptoms at the commencement of the cerebral involvement. In 10 out of the 55 deceased patients the CSF was pathological. In spite of a pleocytosis and the detection of *Listeria*, in 3 cases no pathological changes were found in the cerebrum, probably for the same reason as already mentioned.

According to the above, clinical examination is of limited importance in the diagnosis of listeria meningitis. If symptoms are present, these point to the presence of a meningitis, while the absence of CNS symptoms does not yet exclude it.

In summary, meningitis is frequently observed in connection with neonatal listeriosis. As the clinical symptomatology is not reliable, it is necessary to perform a lumbar puncture in every suspect case.

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LISTERIC MENINGITIS: REPORT OF A CASE

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B. R., a 56-year-old male patient, was admitted on May 8, 1971, in unconscious state with a diagnosis of meningitis. Symptoms of increasing headache and fever began 6 days, sensorial disorders 1 day, before admission. Because of his penicillin allergy chloramphenicol and sulfametin were prescribed. From the day after admission he was artificially respired and tetraclean and erythromycin were substituted for chloramphenicol. After 5–6 days the state improved, the CSF showed a tendency to normalization and the pupillary reaction to light returned. On May 17 the patient died of pneumonia and acute right heart failure.

CSF tests were at admission, on May 13, and May 15: total protein 390, 870 and 330 mg per 100 ml; sugar 50, 105 and 85 mg per 100 ml; cell counts 850, 210 and 130, respectively. In all three specimens lymphoid cells predominated.

Necropsy revealed in the central nervous system demarcating foci not corresponding to the usual picture of purulent meningitis. In addition to a severe liver damage, bronchitis and cystourethropyelitis were observed. As septic conditions causing similar changes were absent, the necropsy supported the diagnosis of listeriosis. Histological examination revealed in the central nervous system granulomes characteristic of listeric meningitis, but failed to show the presence of bacteria.

Bacteriological examination was carried out from the 3 CSF specimens and from the necropsy material. With the exception of the first CSF sample, all specimens were negative either by cultivation or by direct microscopy. The first, greenish-yellow, purulent CSF specimen contained mainly extracellular Gram-positive coccobacilli. In biochemical and cultural behaviour the organism was characteristic of *Listeria monocytogenes*. Injected into mice intraperitoneally, it caused the death of the animals in 5 days; the agent was recovered from the heart blood, spleen and liver. It gave a positive guinea pig eye reaction. Serological examination indicated that it belonged to serotype 4b.

The source of infection remained undetected. The patient was a clerk in Budapest. This was the second verified case of listeric meningitis in Budapest.

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THE PATHOGENICITY OF *LISTERIA MONOCYTOGENES*

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A relatively high carrier rate of *Listeria monocytogenes* has been observed by KILLINGER and HATCH [7] in animals and by BOJSEN-MØLLER [1] and KAMPELMACHER and JANSEN [4] in man. Yet the role of such carriers in transmitting and perpetuating the natural disease is poorly understood. This is especially so in relation to the pathogenicity of the strains, from different sources, including carriers. Indeed, KAUTTER, SILVERMAN and DRAWDY [5] stand alone in reporting the acute toxicity of a number of strains in a single study, using different animals and routes, but they do not indicate the sources of origin of the organisms. More than a decade ago, in 1961 SEELIGER [11] emphasized the desirability of testing the animal pathogenicity of all freshly isolated cultures. Thus a better understanding of listeriosis under natural conditions could be given by looking at the comparative virulence of strains from different sources. The present studies report on the acute toxicity of a number of *Listeria* strains isolated from such different sources and also include their *in vivo* survival in mice.

From the collection of 76 strains 28 (Table I) were selected and maintained on tryptose phosphate agar (TPA) at 4°C and sub-cultured every 3 months. The inoculum was prepared by streaking master tube cultures onto TPA plates incubated for 24 hrs at 37°C. Single colonies were inoculated into 10 ml of tryptose phosphate broth (TPB) and again incubated for 24 hrs at 37°C. Inocula were prepared from this by shaking to give a uniform suspension and preparing 10-fold serial dilutions in tubes of 9 ml TPB. Between the 10-fold dilutions a 2-fold dilution was interpolated so as to give progressive mortality figures which could be subjected to probit analysis to derive the LD₅₀ [3, 6]. Total viable cells per ml of original culture were measured by a pour-plate count to estimate the number of cells per 0.5 ml inoculum in each mouse. The groups of 6 white mice used were of the Schofield (Schofield et Co., Delph, Lancashire) strain (18-22 g at 3-4 weeks) with intraperitoneal inoculation. Infected mice were observed for 10 days with mortality recorded at 12-hr intervals. Postmortem examination was performed on some infected mice by sacrifice or immediately after death and the organs (especially liver

Table I
The LD₅₀ of 22 strains of L. monocytogenes

No.	Strain	Serotype	Source	LD ₅₀	Lambda (standard deviation)
1	L-268	1a	Human carrier	4.1×10^6	0.73
2	L-1012	1a	Human carrier	*	
3	L-1663	1a	Human carrier	4.210×10^6	0.96
4	SB-2	1a	Human patient	*	
5	SB-341	1a	Animal (bovine)	4.3×10^6	0.86
6	SB-369	1a	Animal (ovine)	(8.9×10^5)	(0.54)
7	SB-400	1a	Animal (bovine)	4.5×10^6	0.99
8	COBB	1a	Human patient	(2.8×10^6)	(0.90)
9	NCTC 5105	3a	Not known	*	
10	NCTC 5214	4a	Animal (ovine)	*	
11	NCTC 5214	4a	Animal (ovine)	4.4×10^6	0.77
12	L-252	4b	Human carrier	4.4×10^6	1.22
13	L-994	4b	Human carrier	*	
14	L-1666	4b	Human carrier	4.3×10^6	1.02
15	SBS-1	4b	Silage	(3.7×10^5)	(0.45)
16	SB-611	4b	Animal (bovine)	4.2×10^6	1.10
17	SB-692	4b	Animal (bovine)	3.9×10^6	0.71
18	NZ-1	4b	Human patient	4.1×10^6	1.17
19	NZ-2	4b	Human patient	4.1×10^6	1.51
20	NZ-3	4b	Human patient	3.9×10^6	2.23
21	NZ-4	4b	Human patient	4.3×10^6	1.18
22	NZ-6	4b	Human patient	3.5×10^5	1.07
23	NZ-7	4b	Human patient	4.3×10^6	2.25
24	NZ-8	4b	Human patient	(1.0×10^6)	(0.50)
25	UCH	4b	Human patient	4.4×10^6	1.06
26	Boldy	4b	Human patient	4.0×10^6	1.06
27	C-235	4b	Animal (bovine)	(3.9×10^5)	(0.51)
28	Ox Br	4b	Animal (bovine)	*	

Figures in brackets indicate the LD₅₀ obtained after plotting two points only (mortality below and above 50%).

* Doses more than 10^7 cells failed to kill the mice.

and spleen) were examined for the presence of lesions. For re-isolation and identification of *L. monocytogenes*, tissues were removed aseptically, suspended in 10 ml TPB containing 0.004% (w/v) nalidixic acid and 0.02% (w/v) thallous acetate, homogenized aseptically and sub-cultured on five *solid* selective media: tryptose Negram (TNA; 0.004% Negram nalidixic acid from Winthrop labora-

torics, Newcastle upon Tyne); tryptose thallous acetate Negram (TTNA; 0.02% thallous acetate); Negram potassium tellurite (NPTA; 0.0025% potassium tellurite); along with TPA and blood agars. Subcultures were made by taking one drop with a Pasteur pipette from TPB tube after 48-hr incubation at 30°C. The cultures were examined for morphology, haemolysis and motility with additional fermentation reactions in doubtful cases. Immunofluorescent studies were also made to demonstrate *Listeria* in the tissues of dead and killed mice to compare this technique with the isolation method.

Acute toxicity of 22 diverse strains of *L. monocytogenes* was estimated (Table I). There was little difference in LD₅₀ values between different strains; out of 17 strains for which the LD₅₀ was determined by following regression equations, 16 gave values within the narrow range between 3.9 and 4.5×10^6 organisms. In 5 of the remaining strains, doses of over 10^7 viable cells were non-lethal. One of these (NCTC 5214) only differed from another (NCTC 5214m) by exhibiting a high degree of haemolysis.

Most mice did not appear to be ill until 36–48 hrs postinjection. The first symptoms were (i) the appearance of a ruffled fur, (ii) coldness to the touch, (iii) slight increase in respiration, (iv) a tendency for the eyes to remain closed. During the following 24 hr the eyes filled with a thick muco-purulent discharge which yielded *Listeria*. The mice appeared emaciated — following loss of appetite — and exhibited a mucoid diarrhoea. Most of those infected with large doses seemed to die of overwhelming infection 12 to 24 hr after the appearance of the first symptoms. In some, however, after 4 to 5 days, there was a dragging of one hind limb with partial immobilization of the body and a tendency to lie on one side: all of these died. Some mice, however, which appeared lethargic and showed signs of disease — especially the eye discharge — appeared to recover subsequently. Many of the mice which were dead in 2 to 3 days did not show gross pathological changes in liver, spleen or lung. But those with a longer course of disease showed necrosis of liver and spleen; and the kidney and heart — although not showing visible changes — all yielded growths of *Listeria*. All mice surviving the 10-day observation period for the LD₅₀ assays were retained for *in vivo* survival studies for 15 and 21 days. The effect of the size of the infecting doses for some lethal strains is shown in Table II. Strain Ox Br (for which LD₅₀ was not obtained) gave persistent recovery of *Listeria* from an inoculum of 3.2×10^4 at 15 days but not from 1.5×10^4 at 21 days after infection. Strain SB 611 had an LD₅₀ of 4.2×10^6 cells, but recovery was obtained from an infecting dose of 9.25×10^4 cells, but not from 6.7×10^3 . Similarly the LD₅₀ for NCTC 5214m was 4.4×10^6 , but all organs were harbouring *Listeria* at 15 days after an infecting dose of 3.9×10^5 , though only the liver was infective after 3.9×10^3 cells. Strains L 994 and SB 2 were not lethal at a dose of 10^7 cells, but recovery from the lungs was obtained from doses as low as 4.6×10^5 and 1.05×10^4 .

Table II

Effect of size of infecting dose in the survival of *Listeria* in artificially infected mice 15 and 21 days after infection

Strain	Inoculum	Isolation from						
		Liver		Lung		Spleen		Peritoneal fluid
		15 days	21 days	15 days	21 days	15 days	21 days	21 days
Ox Br	1.46×10^6		+		+		+	+
	3.25×10^4	+		+		+		
	1.46×10^4		—		—		—	
SB 611	6.7×10^5		+		+		+	+
	9.25×10^4	+		+		+		
	6.7×10^3		—		—		—	
NCTC 5214m	3.9×10^5	+		+		+		
	3.9×10^3	+		—		—		
L 994	4.6×10^5		—		+		—	
	3.6×10^5	—		—		—		
	4.6×10^3		—		—		—	
SB 2	3.1×10^7	—		—		—		
	1.05×10^6		—		—		—	
	1.05×10^4		—		+		—	

In a series of experiments on immuno-fluorescence two strains of *Listeria* which were recoverable by culture after 15 and 20 days post-infection could also be demonstrated from the tissues by immuno-fluorescence at 25 and 30 days. The attempts to re-isolate *Listeria* were made by direct cultural techniques and long-term cold pre-incubation was not used. This might have yielded positive results in the cases giving no apparent recovery of *Listeria*.

Mice were susceptible to infection by *L. monocytogenes* although the lethal dose was very high. This gives an impression that the organism has a low degree of pathogenicity for white mice by the intraperitoneal route as compared with e.g. pneumococci, streptococci, or even staphylococci. KAUTTER *et al.* [5] found that both the route of infection and the age of mice were important factors. They found the respiratory route consistent and reproducible with A-4413 and the intraperitoneal route with an LD₅₀ ranging from 19 to 1200 cells in 7 assays. Thus the resistance of the individual may be more decisive in determining the outcome of disease. With exposure in large populations, KAUTTER *et al.* [5] obtained similar results. The host factor was also important and they found that at 3 weeks mice were more susceptible (LD₅₀

10—12 cells) than at 4 weeks (LD_{50} 81—35 cells). Suckling mice were very susceptible (12/16 died with 3 cells).

Early in 1972, COLLINS *et al.* [2] reported some work in the host-parasite relationship of mice with light, chronic infection of *Schistosoma mansoni* super-infected intravenously or orally with *L. monocytogenes*. Listeriae were maintained by regular passage and overnight spleen culture (at 37°C). The intravenous LD_{50} was 5×10^3 . This is in contrast to earlier work by MACKANESS [9] on macrophage activity in which the original virulence of 10^5 cells took 12 months constant passage to reach 5×10^3 intravenously and by MIKI and MACKANESS in 1964 [10] in which the intraperitoneal LD_{50} was 5×10^6 .

Our strains included one (SB369) isolated a week before from a clinical case of sheep listeriosis, yet no significant difference was seen between strains isolated recently, those isolated 1, 2 or 3 years before or those from listeric abortion in women in Auckland (N.Z.). Our results then are in contrast to those of KAUTTER *et al.* [5] — who found LD_{50} 's of 11 to 240,000 cells — and those of COLLINS *et al.* [2] and MACKANESS [8, 9] are more in agreement with those of SEELIGER [11] and MIKI and MACKANESS [10] who reported an intraperitoneal LD_{50} as high as 10^7 cells for white mice. If *L. monocytogenes* has a low virulence, would this not suggest that a higher carrier rate might exist under natural conditions in man and animals — as indeed reported by BOJSEN-MØLLER [1], KILLINGER and HATCH [7], KAMPELMACHER and JANSEN [4] and WILKINSON and HALL [12]. Support for this view comes from the re-isolation and survival of *L. monocytogenes* from mice which appeared healthy in that some of the “high-dose” infected and most of the “low-dose” infected mice seemed to have established a symptomless host-parasite balance. It may therefore be concluded that *L. monocytogenes* can survive in the organs of the mouse challenged with a range of cells.

The positive re-isolation of *L. monocytogenes* after 15 to 21 days, particularly for non-lethal strains, confirms the view of KAUTTER *et al.* [5] that death may be an incorrect criterion for the determination of virulence of *Listeria* strains and that infectivity seems to be a more logical index.

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AN OUTBREAK OF LISTERIOSIS IN DOMESTIC RABBITS

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Listeria monocytogenes was first isolated in 1926 from the peritoneal exudate of a rabbit, then several authors reported on rabbit listeriosis [1-4, 6-8]. In Hungary, it was first observed in 1965 [9]. The causative agent gives rise to meningoencephalitis, septicaemia, abortion and fatal metritis.

In 1971, listeriosis was detected in a rabbit farm, where before sheep were bred that had year by year been affected by listeriosis. Rabbit listeriosis appeared almost unexpectedly, in pregnant rabbits. In one month a total of 53 animals died. At necropsy severe, sometimes purulent, metritis was observed and in the uterus there were mummified foetuses. Most rabbits showed septicaemia with the spleen enlarged 2-3 times and necrotic or inflammatory-necrotic foci in the parenchymatous organs. In 67% of the cadavers there was metritis, in 33% of them septicaemia. No clinical or histological evidence of meningoencephalitis was observed.

From the parenchymatous organs, uterine wall, and foetuses *L. monocytogenes* serotype 1 strains were cultured. The strains were resistant to penicillin, erythromycin and oleandomycin, but sensitive to streptomycin, chloramphenicol, tetracycline and neomycin.

Serological examinations were carried out in a total of 743 sick and suspicious rabbits with serotype 1 antigen prepared by suspending bacteria grown in 0.33% formalin-saline at 37°C for 24 hours. The pH was adjusted to 7-7.4, the density to Brown III. Complement fixation test was not performed because of the frequent anticomplementary effect of the sera. Twenty positive (1:400 or above), 35 doubtful (1:200) and 688 negative (less than 1:200) specimens were recorded. Of the positively reacting animals 65% were males.

In analyzing feeding conditions, it was found that the rabbits were kept on fodder deficient in vitamins A and B.

Oral and parenteral antibiotic treatment chosen on the basis of bacterial sensitivity testing failed to prevent the foetal injury. For half a year the stock was monthly screened for listeria antibodies. Serologically positive females and doubtfully reacting males were excluded from breeding.

The following conclusions have been drawn.

1. Listeriosis may occur in large rabbit farms in epidemic form and may cause considerable mortality.
2. Pregnancy, similarly as in other animals, highly promotes susceptibility to the infection.
3. Bad feeding conditions, especially a deficient vitamin supply, aggravate the course of the disease. Antibiotics had been ineffective until feeding conditions were improved. In eradicating the epidemic, selection of animals by systematic serological screening was helpful.
4. The rabbits had probably been infected from the environment (soil, fodder) responsible also for previous listeriosis among sheep.
5. Serological tests showed that female rabbits usually do not remain carriers of *Listeria*, as they die almost unexceptionally in the late stage of pregnancy. Serological tests are especially recommended for animals which carry the organism without symptoms. Carriership can be detected with repeated serological tests.

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PATHOGENICITY OF *LISTERIA MONOCYTOGENES* IN THE RAT

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Adult (over 200 g) male and non-pregnant female white rats were found to be resistant to *Listeria monocytogenes* infection, since 1.0 ml of a 20-hr broth culture (approx. 3×10^7 organisms) introduced subcutaneously, or in the femoral muscle or in the abdominal cavity failed to cause death. The result of the intraperitoneal injection was slight septicaemia for a few days and no titre was found in the serum of surviving animals (Table I). On the other hand, in animals with a pregnancy of more than 7 days the intramuscular injection of 10^6 to 10^7 bacteria (0.2 to 0.5 ml broth culture) caused in every instance foetal death. No symptoms were observed in the rats which had been infected when 7 to 12 days pregnant, while those infected between 9 to 14 days of gestation displayed prostration, ruffled fur and loss of weight, besides the

Table I

Result of intraperitoneal L. monocytogenes infection of non-pregnant adult rats
(Infecting dose 1.0 ml broth culture*)

Infected rats		Isolation of <i>L. monocytogenes</i>					Reciprocal agglutination titre in serum
No. of animals	Time of killing, day after infection	Abdominal cavity	Urine	Blood	Liver	Spleen	
6	2	5**	4	2	2	4	20 (1)***
2	3	1	1	—	1	—	
5	4	—	1	1	2	3	
8	6	—	—	—	2	2	
22	8	—	—	—	3	2	20-40 (3)
37	10-12	—	1	—	2	1	20-40 (3)

* Virulent 1/2a type *L. monocytogenes* isolated in spring, 1970, from the brain of a sheep died from encephalitis

** In 5 cases out of the sacrificed animals

*** No. of animals showing the titre indicated

resorption of the conceptus (Fig. 1). Under the effect of such massive doses both the mothers and their foetuses died, if the pregnancy was older than 12 to 14 days. Fig. 1 indicates that, similarly to the non-pregnants, massive infection failed to exert a harmful effect on animals with pregnancy of less than 6 days and on those being immediately before delivery. Infection of

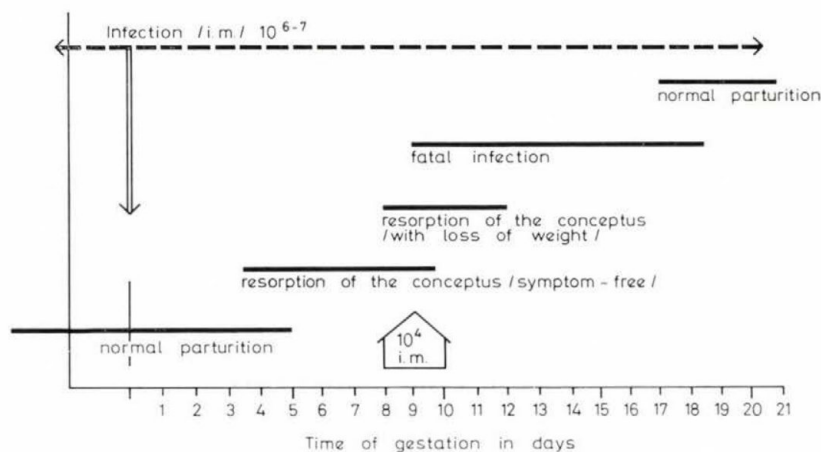


Fig. 1. Damage in rat mothers and foetuses caused by experimental listeriosis

9 to 12 days pregnant animals with 10^4 organisms resulted in abortion and premature delivery. In these cases characteristic necrotizing foci (listeriomaes) were demonstrable in the liver of the expelled foetuses and also in some living newborns.

Oral infection was unsuccessful even in animals with half-time pregnancy. This might explain the fact that under natural conditions *L. monocytogenes* causes no foetopathy in rats.

On the other hand, newborn rats could successfully be infected orally. One day old animals displaying the highest susceptibility were chosen for this purpose, and infection was carried out by introducing orally 1 to 2 drops of broth culture with a pipette (Table II). Animals infected one day after birth became anaemic and lagged in development within 2 to 5 days, they scarcely moved and nearly 100% of them died with necrotic foci in the livers. On infecting two-day-old suckling animals, half of the litter died similarly, the lag in development became, however, more expressed. Oral infection of older suckling animals had a weaker pathogenic effect. In 8 to 10 days old suckling rats it failed to cause illness; and only the presence of listeriae, being demonstrable for about two weeks in the faeces of the animals, referred to the infection. Similar results were obtained with rats which had been infected at a young age and survived the infection.

Table II

Result of oral L. monocytogenes infection of newborn rats
(Infecting dose 1 to 2 drops of broth culture*)

Newborn rats		Death, per cent	Retarded animals, per cent
Age	No. of litters		
1 day	15	70—100	0—30
2 days	14	30—50	50—70
3 days	8	22	50
5 days	5	6	15—30
10 days	6	0	5—15

* See Table I

Introducing the pathogen under the dorsal skin of rats weighing 25 g and of suckling rats of lower weight, the animals succumbed with bacteraemia similarly to the far more susceptible adult white mice. In several cases nodes of plum-stone size were found under the dorsal skin at the site of infection. Fatal listeriosis developed in part of rats of more than 25 g weight (Table III), especially in those which had been weaned early. In rats of 75 g weight, subcutaneous infection failed to induce listeriosis.

Table III

Result of subcutaneous L. monocytogenes infection
of suckling and young rats
(Infecting dose 0.2 to 0.5 ml broth culture*)

Weight group	No. of animals	Death, per cent	Retarded animals, per cent
10—15	72	100	0
16—25	48	72—90	10—28
26—50	58	approx. 50	50
51—100	63	8	70—90

* See Table I

Intraperitoneal infection of animals of 75 g or less weight caused in nearly 100% disease or death. Infection of rats weighing 100 g was lethal in 50% (Table IV). In adult animals of 200 g weight it was almost impossible to induce fatal disease, even by intraperitoneal inoculation.

Antibodies were found only in the sera of diseased but surviving young rats infected by the subcutaneous or the intraperitoneal route. The titres observed were between 1 : 80 and 1 : 320. Superinfection induced partial im-

Table IV

*Results of intraperitoneal L. monocytogenes
infection of young rats*

(Infecting dose 0.5 to 1.0 ml broth culture*)

Weight groups	No. of animals	Per cent of death	Per cent of retarded animals
under 50 g	81	100	0
51 to 75 g	66	70—86	14—30
76 to 100 g	50	45—60	approx. 50
101 to 150 g	48	20—45	approx. 20
151 to 200 g	49	8	approx. 10

* See Table I

munity. This was shown by an experiment carried out in 16 young animals which survived a subcutaneous infection carried out three weeks before, and after superinfection in the abdominal cavity only 4 of them died, on the other hand, out of 36 control animals of the same weight and infected with the same dose, 32 died from listeriosis.

These experiments revealed that young animals are far more susceptible to *L. monocytogenes* than adult ones. Foetuses and newborn rats are particularly susceptible. Perinatal oral infection induced the same pathological changes as could be found in older foetuses which had become infected and died *in utero*. It seems that it is due to this resistance of adult rats that naturally occurring listeria infection seldom causes foetopathy, abortion and premature delivery in this species.

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IMMUNOGLOBULINS IN SHEEP INFECTED WITH LISTERIA STRAINS

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Studies of the immunoglobulins in listeriosis have been made by AALUND *et al.* [1], OSEBOLD and AALUND [2] and SEELIGER and EMMERLING [3], who established the significance of IgG in infected animals.

We have studied the dynamics and appearance of IgM and IgG in the blood serum of sheep experimentally infected with *Listeria monocytogenes*. For this purpose, 12 sheep were divided into two groups of 6 animal each of which one group was infected with 24 hr broth cultures of *L. monocytogenes* of serogroup 1 in such a manner that 3 sheep received 2 ml of the culture intravenously and the other 3 received 5 ml subcutaneously. The sheep in the second group were infected according to the same method with *L. monocytogenes* type 4b. Before the infection, tests for the detection of antibody were made two times using the agglutination reaction (SAT), complement fixation test (CF) and indirect haemagglutination test (IHT). From the second day on samples were obtained every week for 14 weeks. In the main experiment, IHT was performed before and after treatment of the serum with 2-mercaptoethanol (ME) according to MORGAN [4]; to each dilution of serum 0.05 ml of 0.2 M ME was added and after standing for 16 hrs at room temperature, sensitized erythrocytes were added. The highest dilution in which agglutination was observed was taken as the final titre (++). In the preinoculation serum of some sheep, ME-labile antibody was detected in low titre, 1 : 40. On the second day after infection, ten sheep showed titres of 1 : 10 to 1 : 160, which were due to antibody labile to ME. During the following days, the titres showed a tendency to increase and reached their maximum between 13 to 34 days (see Table I).

After this period, they began to decline, but remained at a relatively high level up to the 97th day, when the experiment was terminated. The ME-stable immunoglobulins appeared on the 7th day and their titre increased gradually in the period between 13–34 days, a levelling of the titres of both types of antibody occurring in different animals at different moments. In four of the animals the titres were represented by the ME-resistant antibodies for a period of 7–18 days; in the remainder, the period was much shorter. After

Table I

Immunglobulins in listeriosis in sheep studied by means of the IHT

No. of sheep		Titres before infection	Titres on days after infection																
			2	4	7	10	13	16	20	22	27	34	41	48	56	63	75	90	97
14	IgM IgG	— —	10 —	40 —	10 —	40 —	10 —	20 20	20 20	20 20	40 10	160 —	— —	20 —	20 —	20 —	80 10	— —	320 20
15	IgM IgG	40 —	40 —	80 —	— —	10 —	20 —	80 —	20 20	20 20	80 80	5120 10	80 20	160 —	80 —	160 20	40 —	320 20	320 20
16	IgM IgG	40 —	40 —	80 —	40 —	40 —	160 80	80 40	80 40	80 80	80 40	1280 40	640 20	640 20	160 10	320 10	80 20	80 80	320 40
17	IgM IgG	— —	— —	40 —	— —	320 —	40 20	160 160	80 80	80 80	160 160	80 80	640 20	640 —	160 40	640 40	320 20	320 80	+ +
18	IgM IgG	20 —	40 —	160 —	160 —	160 —	80 —	160 40	40 40	160 80	80 40	80 40	640 40	640 40	160 20	320 20	160 20	320 40	80 80
19	IgM IgG	10 —	20 —	80 —	40 —	40 —	80 —	80 40	160 40	160 160	80 80	40 40	640 40	640 40	160 40	640 40	80 20	320 40	320 40
11	IgM IgG	40 —	40 —	20 —	640 160	640 320	2560 640	1280 1280	2560 1280	2560 640	1280 640	640 640	160 20	320 40	160 20	320 20	320 20	320 20	80 20
12	IgM IgG	10 —	20 —	10 —	640 160	640 20	320 320	640 640	1280 1280	1280 640	320 320	160 160	320 20	80 20	80 20	80 20	10 20	320 20	160 20
13	IgM IgG	— —	— —	— —	160 80	640 320	2560 320	1280 1280	1280 640	320 160	640 640	640 320	80 10	160 40	160 40	80 20	320 20	320 20	320 20
20	IgM IgG	10 —	10 —	20 —	640 320	2560 640	640 640	1280 160	5120 2560	640 320	1280 320	640 40	160 20	1280 40	160 20	160 40	320 40	320 40	320 40
21	IgM IgG	10 —	10 —	20 —	640 320	640 80	640 160	320 320	1280 160	160 80	2560 —	1280 80	80 —	160 10	40 —	40 20	320 40	320 40	80 20
22	IgM IgG	— —	— —	20 —	160 80	1280 320	2560 80	1280 1280	5120 2560	2560 640	5120 40	640 80	640 80	320 40	160 40	320 40	320 40	320 40	320 (

the 34th day, the titre of IgG, present in every animal until the end of the observation (97 days) was some dilutions lower than that of IgM.

Difficulties in the serological diagnosis of listeriosis are encountered due to the existence of common antigenic determinants between *L. monocytogenes* and some wide-spread Gram-positive microorganisms such as enterococci, staphylococci, corynebacteria and others. RANTZ *et al.* [5] discovered in some

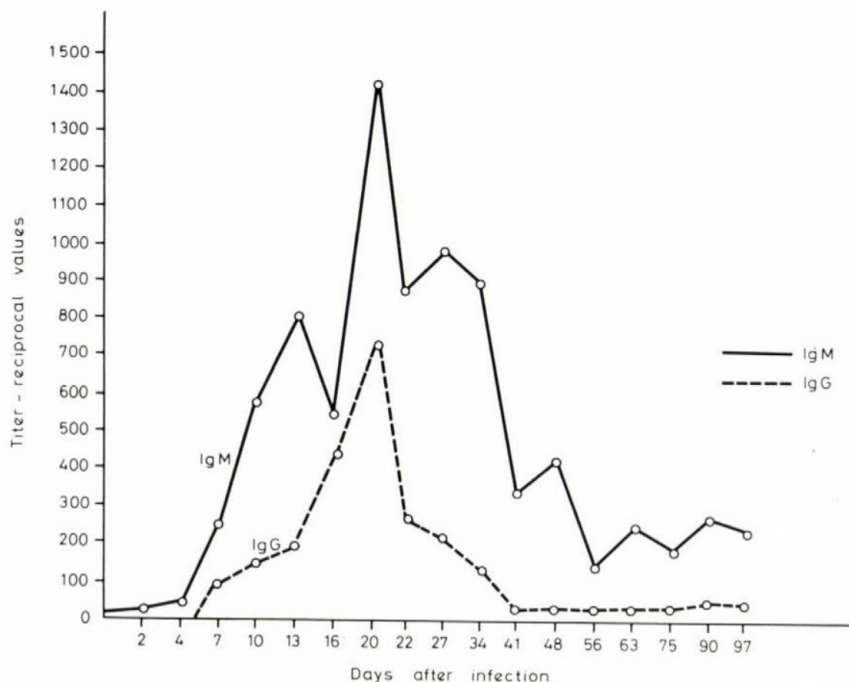


Fig. 1. Mean titres of IgM and IgG

Gram-positive bacterial species non-specific antigens, which NETER *et al.* [6] observed also in *L. monocytogenes*. AALUND *et al.* [1] and OSEBOLD and AALUND [2] studied sera from infected sheep and cattle and from those with normal antibody, before and after treatment with ME, and found that cross-reactions appear in animals stimulated with antigens which have common determinant groups and are found mainly with IgM antibodies. The serum from animals with demonstrated infection reacted after ME treatment only with homologous antigens. The said authors considered the reaction sufficiently specific for clinical and epizootological studies. However, our results showed that in the early period of infection, the infected animals reacted only by producing IgM antibody; thus accepting that all animals reacting by producing IgM immunoglobulins are healthy carriers presents a serious risk. Since the limit of the

appearance of IgG antibody in experimental infection is 7 to 16 days, investigation of animals suspicious of infection at least twice at 15 day intervals is a necessary condition for discovering real infections. The test facilitates exact diagnosis since IgG immunoglobulins persist for a long period parallel with IgM immunoglobulins. According to SEELIGER and EMMERLING [3], IgG antibody, not only against O antigen, but also against H antigen of listeriae, is found in the limit of "normal" antibody. In this case, it is of considerable importance to estimate the "normal" and pathological titres reliably. In our experiments, the preinoculation titres of the animals were 1:40 and they were represented exclusively by ME labile antibody. During the process of infection we constantly found IgM as well as IgG antibody independently of the fact that in some cases the titre of IgM was under the pathological limit. This might have a significance in tracing animals with inapparent infection, or in those treated with antibiotics, in which cases the antibody level is always below the pathological titre.

Independently of the mentioned difficulties, the mercaptoethanol test allows significantly to improve the serological diagnosis of listeriosis, by enhancing the specificity of the reactions. With the use of this test, indirect haemagglutination is preferred to agglutination since, according to SEELIGER and EMMERLING [3], cases with incomplete (IgG) antibody can be discovered. We have observed its high sensitivity during the whole course of the experimental period.

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ACTIVE IMMUNIZATION WITH FORMOL VACCINE AGAINST SHEEP LISTERIOSIS

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Listeriosis usually occurs in sheep between December and May [1, 8]. As this observation indicated that the appearance of the disease may depend on several factors, three different measures had to be considered: immunization, antibiotic treatment and improvement of foddering.

As sick sheep usually fail to react to therapy [4, 5, 7], little effect was expected from the application of antibiotics. However, we thought that the addition of antibiotics to the fodder would prevent the development of clinical symptoms in sheep not immunized actively. On the other hand, in view of the possible impairment of the rumen flora and development of antibiotic resistance in certain bacteria, a prolonged feeding of antibiotics was contraindicated. Since literary data showed the effectiveness of antibiotic treatment [2], the experiment was performed on the basis of antibiotic sensitivity testing of the agent *in vitro*.

Two drugs were applied: ERRA-6 containing 60 g oxytetracycline per kg and Ticofuran containing 30 g 5-nitro-2-furfuryldene per kg. The experiment was performed in a farm where a considerable number of sheep suffered from listeriosis.

Approximately 2500 sheep were kept in 7 pens situated side by side. The outbreak started parallel with lambings in December, when 31 cases were observed. The measures applied are shown in Table I. The animals were divided into groups A—G. Half of the animals in each of groups A, B and C received 5 ml formalinized listeria vaccine twice at 2 week intervals subcutaneously. The vaccine was prepared with serotype 1/2 strain "B" chosen from 177 cultures isolated by us. This strain had the highest pathogenicity and the best immunizing power in laboratory experiments.

Group D received neither immunization nor antibiotic treatment. Group E was given Ticofuran (1.5 kg per 100 kg fodder), group F oxytetracycline (1 kg ERRA-6 per 100 kg fodder). Group G received no silo fodder, on which sheep are usually fed in the winter.

In comparison to control group D, the incidence of listeriosis was similar in groups E, F and G and in non-vaccinated animals of groups A, B and C. Among immunized sheep the infections occurred less frequently.

Table I
Immunization of sheep against listeriosis

Groups and No. of animals	No. of deaths with listeriosis					
	January	February	March	April	May	Total
A 50% vaccinated	2	—	—	1	—	3
400 50% control	12	7	2	5	—	26
B 50% vaccinated	2	1	—	—	—	3
360 50% control	9	10	3	—	—	22
C 50% vaccinated	1	—	—	—	—	1
200 50% control	18	15	1	5	—	39
D						
200 Control	14	10	16	6	3	49
E 100 kg fodder + 500 1.5 kg Ticofuran	18	5	11	8	—	42
F 100 kg fodder + 500 1 kg ERR-6	11	15	9	9	1	45
G No silo fodder given 500	18	12	12	7	—	49

During the fermentation of silo fodder the low incubation temperature may be advantageous for the multiplication of listeriae. Examination of many hundreds of silo samples, however, failed to show the presence of the organism either by cultivation or by animal inoculation, although many specimens had been taken from farms where listeriosis was prevalent.

Our findings disagree with the theory of certain authors [3, 6] that listeriosis can be prevented by discontinuing silo fodder feeding.

Our 10 years experience with the immunization of about 10,000 sheep indicates that vaccination is the most effective measure against sheep listeriosis. The first injection should be given in the 3rd month of pregnancy, the second 2—3 weeks before lambing. In this manner infection of the sensitive and unvaccinated lambs can usually be prevented.

Experience in other farms has shown that, in addition to vaccination, it is advisable to let the animals walk every day whenever the weather allows it. Fodder rich in vitamins and early beginning of pasturing are also beneficial. At artificial regulation of the time of lambing, the most dangerous season should be avoided.

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ABORTION IN GRAY'S MONKEY (*CERCOPITHECUS MONA*) ASSOCIATED WITH *LISTERIA MONOCYTOGENES*

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Listeriosis seems to occur infrequently in monkeys and there is only one paper which deals with listeria infection in a marmoset [1].

In the Budapest Zoo, a 10-year-old Gray's monkey (*Cercopithecus mona*) weighing 3.5 kg aborted an almost completely developed (about 5-month-old) foetus. The abortion started unexpectedly and ended without complication. At necropsy the foetus 22 cm in length and 200 g in weight showed a slight degree of maceration and the subcutaneous connective tissue was imbibed with serofibrinous fluid. The placenta weighing 50 g was connected with the foetus by an anaemic umbilical cord. The placenta had greyish-white spots on its surface and section. The aborted foetus originated from the 5th pregnancy of the monkey (Table I). While this paper was being written, the monkey has again become pregnant.

Table I

Pregnancies of a female Gray's monkey (Cercopithecus mona)

Pregnancy	Result
1. May 5, 1965*	1 healthy offspring
2. February 14, 1966	1 healthy offspring
3. March 20, 1967	1 healthy offspring
4. January 5, 1969	1 healthy offspring
5. January 29, 1972	1 dead foetus
6. ?	?

* At this time the monkey was 4½ years old

Bacteriological examination yielded listeriae from the liver, lung, brain and kidney of the foetus. The placenta contained these bacteria in relatively low numbers. The organism was identified as *Listeria monocytogenes* serotype 4b. It killed white mice after intraperitoneal injection in 2-4, after subcutaneous injection in 3-7 days. On the basis of previous experiments [2] 1.0 ml of its broth culture was given orally to two pregnant rabbits. The ani-

mals died with septic metritis 12 and 14 days after the infection. Oral infection of one-day-old rabbits resulted in fatal septicaemia. Three weeks after the abortion the serum of the mother monkey agglutinated the corresponding listeria antigen at 1 : 160 titre, which, according to experience in human listeriosis could not be regarded as of diagnostic value.

Histological examination indicated septicaemia in the foetus. In the liver parenchyma and in the lung there were submiliary, multiple necrotic and necrotic-inflammatory foci. In the liver the majority of foci were purely necrotic in type. Certain foci showed RES cell proliferation. The fibre network of the liver disintegrated at the site of the foci. Independently of the foci, the liver cell rows were separated by serous exudate. In the lung there was an interstitial mononuclear infiltration around multiple inflammatory foci. In certain alveoli, aspiration of amniotic fluid and epithelial cells were seen. In the foetal placenta both the subchoroidal and decidual edge showed the presence of focal necrotic-fibrinous-purulent inflammation. Necrotic chorionic villi were stuck together by fibrin, the wall of chorionic capillaries was loosened, the chorionic epithelium (trophoblast) disintegrated and thus the mother's blood mixed with the foetal blood. In the lumen of certain capillaries fresh parietal thrombi containing listeriae in great numbers were observed. The amniotic epithelium was loose at some sites but the basal amniotic membrane remained mostly unchanged.

In the liver, mainly phagocytized bacteria were revealed by the modified Gram stain. The serous exudate in Disse's spaces also contained listeriae.

The lung (capillaries, bronchi and alveoli), the brain (axis), the kidney (mainly the medullary substance), the heart (capillaries and spaces between muscle cells), lymph nodes, umbilical veins, cytoplasm of amniotic epithelial cells at the site of the foci showed the presence of great numbers of bacteria.

The placenta contained only a moderate number of degenerated listeriae. Bacteria were especially infrequent where neutrophil leucocytes predominated.

It has been concluded that in the mother monkey the infection caused pathological changes in the placenta (mainly at the site of the subchorial sinus and of the decidua). The foetus was infected from that site, and the amnion from the foetus. Changes in the foetal lung were due partly to septicaemia, partly to an aspiration of the infected amniotic fluid.

The source of the infection could not be detected. Neither the organs or the faeces of 26 wild rats trapped at the monkey house nor the faeces of the mate of the monkey yielded listeriae. It may be assumed that some of the foodstuff (vegetables, salade, rhubarb, dairy products) given to the monkeys contained the organism.

The present case was interesting mainly from the point of view of comparative pathology, and at the same time it calls attention to the monkey as a possible model for studying human listeriosis.

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Closing Address by Professor H. P. R. Seeliger

In a closing address Professor SEELIGER thanked in the name of the foreign participants for the warm and cordial hospitality offered by the organizers of the Meeting.

Among others, he said: "The Scientific Meeting gave an excellent review of the present state of knowledge and offered ample opportunity for exchanging views on many problems. It appears that better antigens are needed for evaluating with more success the immunological response in animals and man. The views expressed on this topic were in some ways conflicting and did not allow epidemiological conclusions. This was underlined by reports on the isolation of *Listeria* strains from soil, vegetable matter and faecal specimens. Since quite a number of the latter strains show biochemical and serological reactions slightly different from the well-known pattern of *Listeria monocytogenes*, more studies seem to be needed as to their taxonomic position and ecology. The absence of pathogenicity in many of these strains requires further studies. As a matter of fact one may have to deal with more than one species originally thought to be *L. monocytogenes*. This would possibly include the so-called serotype 5 with its pronounced beta haemolysis. More work is needed for applying listeria vaccines for the protection of animals at risk and certainly more attention has to be paid to this organism in hospital wards, intensive care units and centres for haemodialysis. In consequence a great deal of work lies ahead and it is hoped that numerous members of the International Listeria Community can meet again in Nottingham, England, where Dr. WOODBINE will convoke the next International Symposium on Listeriosis in 1974 (or 1975?)."

Professor SEELIGER concluded his address by pointing out the excellent spirit which became apparent during the discussions at both the scientific and the personal level. "I am" — he continued — "of the firm opinion that international meetings of such quality will greatly contribute to a better understanding between individuals, nations and systems of society. The greatest achievement is, however, the possibility to acquire more knowledge essential for every scientist as long as he lives."

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